TISSUE FACTOR INDUCTION AND REGULATION IN MONOCYTES

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DECLARATION

In accordance with postgraduate degree regulation 3.8.7 of the University of Edinburgh, I declare that the work described in this thesis is my own, except where otherwise indicated in the text, and that it has not been submitted for any other degree. I also acknowledge all collaborations and assitance given to me during the course of these studies.

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ABSTRACT

Tissue Factor (TF; CD142) is the principal initiator of the coagulation cascade. This 45kDa protein is constitutively expressed in extravascular cells and as such forms a protective haemostatic envelope surrounding the vasculature ready to initiate coagulation on vascular injury. When bound to its cofactor factor VII/VIIa in the normal bloodstream, it forms a highly catalytic complex (TF/VIIa) which triggers the activation of factors IX and X and ultimately the formation of a fibrin clot. Peripheral blood cells do not, in a resting state, express TF allowing maintenance of the fluidity of the blood. However, agonists such as bacterial lipopolysaccharide (LPS) and immune complexes, if present in the bloodstream can induce the expression of TF on monocytes resulting in activation of the clotting process. This induction of monocyte TF has been clearly implicated in the pathology of various disease processes such as disseminated intravascular coagulation (DIC) following endotoxaemia, thromboembolic disease and atherosclerosis. I have characterised a whole blood model to investigate the induction of TF on monocytes, and have developed a highly specific and sensitive flow cytometric technique to measure the TF protein on the cell surface of activated monocytes. I have shown that there is a reproducible dose dependent induction of TF on monocytes in whole blood by LPS (10pg/ml-100μg/ml) after a 2 h incubation. Antisense therapy is aimed at the specific inhibition of a particular target protein by preventing the translation of its mRNA. Antisense (AS) oligodeoxynucleotides (ODNs) are short single stranded lengths of reverse complementary DNA which can be designed to specifically hybridize to a target mRNA, through Watson and Crick base pairing laws, arresting translation of the protein. I have investigated the potential of ASODNs to inhibit specifically the nascent induction of TF in monocytes in response to LPS. I have characterised the uptake kinetics of a fluorescently labelled 25 base phosphorothioate oligonucleotide GEM-91 (Gene Expression Modulator number 91) into peripheral blood leukocyte subtypes. I have shown that the uptake of GEM-91 is dose and time dependent and is not saturated at concentrations of up to 10μM for 4 hours. I have also shown that monocytes internalize 2 and 4 fold higher levels of oligonucleotide than neutrophils or lymphocytes respectively. Monocytes internalized approximately 200fg GEM-91/cell after 0.5 hours in culture rising to 800fg/cell after 4 hours. This apparently selective and efficient uptake of ODNs in monocytes suggests that they may be an attractive target for antisense therapy. It has been suggested that ODNs enter cells by the active process of receptor mediated endocytosis. A number of groups have alluded to the identity of the putative antisense receptor. I have investigated the involvement of two monocyte cell surface receptors in the uptake of ODN, and have concluded that both the Mac-1(CD11b/CD18) and CD14 receptors may be involved to differing extents in the uptake of ODN into monocytes. I then went on to investigate the potential of three different antisense ODNs to TF to reduce the induction of TF by *E. coli* O111:B4 LPS in monocytes in whole blood. I have shown that certain ODNs actually induce TF on monocytes and that as a result an equilibrium may exist between TF induction by ASODNs and its inhibition. I have concluded that ASODN offer great potential as an anti-TF therapy in monocytes, but much work needs to be done to improve their specificity, delivery and intracellular bioavailability. The TF inducing ability of a range of LPS molecules from different bacterial sources

has also been investigated. The experiments described have shown that LPS structure and bacterial source are important in determining the potency of LPS at inducing TF. In addition, I have concluded that LPS from the ubiquitous gut bacterium *Bacteroides fragilis* is less potent at inducing TF than *E. coli* LPS and have also shown that this LPS is capable of reducing the potency of the *E. coli* LPS at inducing TF. This may have protective implications. In summary, the studies reported in this thesis aimed to investigate the expression and control of TF in peripheral blood monocytes. The therapeutic potential of ASODNs at inhibiting this expression has been investigated. Particular attention has been paid to the uptake kinetics of ASODNs into peripheral blood leukocyes and the possible mechanisms involved. The possible protective effect of one bacterial LPS against the toxicity of another has also been investigated.

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ABBREVIATIONS

aFGF	Acidic fibroblast growth factor
AS	Antisense
ASODN	Antisense Oligodeoxynucleotide
bFGF	Basic fibroblast growth factor
bp	base pair
cAMP	Cylic Adenosine Monophosphate
COS	Cells derived from an african green monkey kidney cell line
CPB	Cardiopulmonary Bypass
CQ	Chloroquine
cv	Coefficient of Variation
DIC	Disseminated Intravascular Coagulation
DNA	Deoxyribonucleic Acid
E. coli	*Escherichia coli*
EDC	1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride
EDTA	Ethylenediaminetetraacetic Acid
GEM	Gene expression modulator
GMF	Geometric Mean Fluorescence
FACS	Fluorescence Activated Cell Scanner
FAM	Fluorescein Addition Monomer
FITC	Fluorescein IsoThioCyanate
FSC	Forward Angle Scatter
HETE	12-Hydroxy-eicosatetraenoic acid
HeLa	Epithelial-like cell line (Helen Lane, the patient)
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM-1	Intracellular Adhesion Molecule-1

IgG	Immunoglobulin
IL	Interleukin
kDa	Kilo Dalton
Kdo	3-deoxy-D-manno-2-octulosonic acid (2-**k**eto-3-**d**eoxy**o**ctonate)
LDL	Low Density Lipoprotein
LPS	Lipopolysaccharide
mAb	Monoclonal Antibody
MEG	Reverse sequence of GEM-91
mRNA	Messenger Ribonucleic Acid
MDCK	Madine-Darby Canine Kidney
NF-κB	Nuclear Factor Kappa Beta
NK	Natural Killer
ODN	Oligodeoxynucleotide
PAF	Platelet Activating Factor
PBL	Peripheral Blood Leukocyte
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PDGF	Platelet Derived Growth Factor
PI	Propidium Iodide
PL	Phospholipase
PMA	Phorbol myrisitate acetate
PMN	Polymorphonuclear Leukocytes
PPP	Platelet Poor Plasma
PRP	Platelet Rich Plasma
PS	Phosphatidylserine
PE	Phycoerythrin
RAMPE	Rabbit Anti-Mouse Phycoerythrin
sem	standard error of the mean
SSC	Side Angle Scatter
TF	Tissue Factor

TFO	Triplex Forming Oligodeoxynucleotide
TFPI	Tissue Factor Pathway Inhibitor
THP-1	Human monocytic cell line
TKO	Transgenic Knockout
TNF-α	Tumour Necrosis Factor Alpha
TPA	Tissue Plasminogen Activator
VEGF	Vascular Endothelial Growth Factor

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For my parents, Sean and Bernadette McIlroy.

CHAPTER 1

INTRODUCTION AND BACKGROUND

1.1 Historical Perspective

The ability of tissues, or their extracts, to induce the clotting of blood was first reported by Thackrah in 1819 and was later confirmed by de Blainville in 1834 when he noted that the infusion of a suspension of brain into animals produced widespread coagulation within blood vessels and subsequent death. Schmidt (1892) believed that the tissues contained a substance that directly converted prothrombin to thrombin resulting in clot formation. Consequently, much of the work done in the early 1900s was concerned with identifying the nature of this procoagulant activity present in the tissues. It was argued that the responsible substance was either a lipid, a protein, an unknown activator of prothrombin, or thrombin itself (Howell, 1912; Mills, 1921; McLean, 1916). In 1921, as part of his doctoral thesis, Mills sites Wooldridge as having demonstrated that the procoagulant activity of tissues required both phospholipid and protein and was thus due to a lipid/protein complex. In his research, he went on to demonstrate that the protein and lipid fractions of tissues could be separated, resulting in loss of procoagulant activity, but that these components, when recombined, possessed 250-fold more activity than the individual fractions. Subsequent studies went on to demonstrate that the potent procoagulant activity in tissues was an apolipoprotein (Chargaff, 1944,1948) and that it required both a protein and a phospholipid portion for full procoagulant activity (Kuhn and Cleese 1957; Hvatum and Prydz, 1969; Deutsch *et al.*, 1964, Nemerson, 1968). It is now accepted that the potent procoagulant property of tissues is due to tissue factor (TF) (factor III, thromboplastin) or more accurately the tissue factor apoprotein complexed with membrane phospholipids (Carson, 1984; Mann *et al.*, 1988; Bach, 1988; Nemerson, 1988; Broze, 1992; Roberts, 1992).

1.1.1 The Coagulation System

In 1905, Morawitz summed up the previous century's work leading to a theory of blood coagulation involving the interaction of four factors. Three factors prothrombin, calcium ions and fibrinogen, were present in plasma whilst the fourth,

thrombokinase (tissue factor) was believed to be contained within the platelets and leukocytes. When blood came into contact with tissues, platelets and leukocytes were thought to aggregate and liberate TF. The released TF was then thought to react with prothrombin in the presence of calcium ions to generate thrombin which converted fibrinogen into the fibrin strands of a blood clot. Destroyed cells were believed to provide a second source of TF , which caused the blood to clot more rapidly at the site of a wound. By the middle of the century, as new clotting factors were discovered, it became apparent that blood coagulation could be initiated in at least two ways. Finally in 1964, Macfarlane and Davie & Ratanoff had developed the cascade hypothesis of coagulation, separating the known clotting factors into two pathways; the intrinsic and the extrinsic converging at the activation of factor X. In the intrinsic pathway the exposure of the contact factors (factor XII, high molecular weight kininogen and prekallikrein) in plasma led to the activation of factor XI with susequent activation of factor IX which, in the presence of factor VIII cleaved factor X to factor Xa. In the extrinsic pathway, coagulation was triggered by the binding of plasma factor VII to TF, with susequent activation of factor Xa. Factor Xa then converted prothrombin to thrombin, which induced the formation of cross linked fibrin from fibrinogen monomers and finally a blood clot.

1.1.2 TF /TFPI in Haemostasis

The catastrophic bleeding of haemophiliacs, who are deficient in factor VIII but have an intact extrinsic pathway initially led investigators to believe that the intrinsic pathway initiated coagulation during haemostasis. However, the fact that patients deficient in one of the contact factors required to initiate the intrinsic system are asymptomatic (Hathaway *et al.*, 1965; Nemerson & Furie 1980; Kaplan & Silverberg 1987) whereas individuals deficient in factor VII bleed abnormally (Ragni et al 1981; Triplett et al 1985), and the obsevations by Østerud & Rappaport (1977) that factor VIIa/tissue factor can activate factor IX of the intrinsic pathway as well as factor X led to a resurgence of interest in the extrinsic pathway in the 1980s. The recent discovery of an endogenous inihibitor of TF, Tissue Factor Pathway Inhibitor (TFPI),

a novel, endogenous, kunitz-type inhibitor of TF mediated coagulation, led to the reorganisation of the system placing TF as the prime initiator of coagulation. The first step which occurs upon the exposure of blood to extravascular tissues is the binding of VII to TF, its cell surface receptor and cofactor. The resulting TF/factorVII complex is activated by trace amounts of factor Xa present in blood or the extravascular space, forming the highly active enzyme complex TF/factorVIIa. This in turn activates both factor IX and factor X. Factor Xa then converts prothrombin to thrombin, which finally induces the formation of fibrin from fibrinogen monomers resulting in a blood clot (Fig. 1.1). Consequently, today coagulation is divided into two stages rather than two pathways: an 'initiation' stage which is handled by the TF-dependent pathway, and an 'augmentation' stage, which is handled by the components of the previous intrinsic pathway. Both pathways are able to generate fibrin, but the TF pathway is shut off by TFPI, soon after initiation. By this time, however, sufficient thrombin will have been generated to activate factors V, VIII, and XI. The second augmentation stage of the pathway will then ensure prolonged activation of the coagulation cascade (Fig 1.1). This model suggests that TF is responsible for activation of the coagulation cascade whilst the intrinsic components are required for sustaining haemostasis, underlining the rationale behind TF inhibition as an anti-thrombotic therapy.

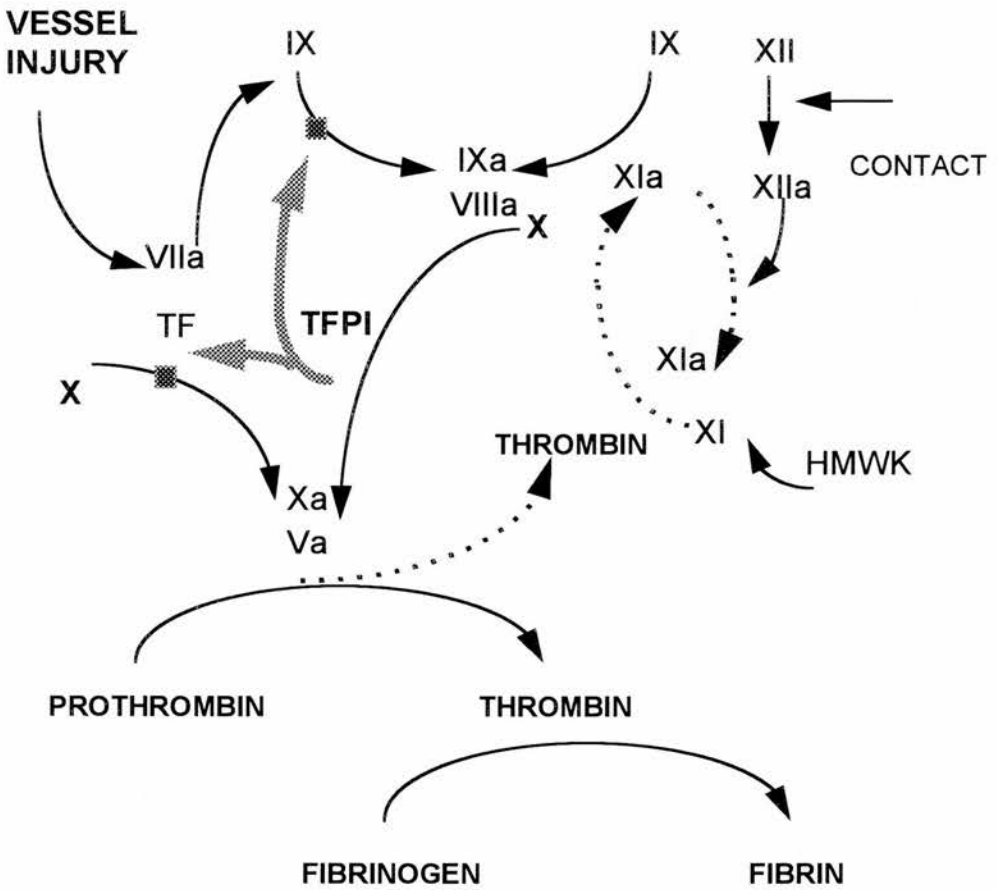


FIGURE 1.1 The Role of Tissue Factor in Coagulation
Haemostasis is initiated when factor VII or factor VIIa comes into contact with TF at a site of blood vessel injury. Limited quantities of factor IXa and factor Xa are generated before there is feedback inhibition of the factor VIIa/TF complex mediated by TFPI and factor Xa. The generation of factor Xa is then amplified through the action of factor VIIIa and factor IXa; the latter produced initially by factor VIIa/TF and supplemented by factor XIa. Factor XI activation may be produced by thrombin and autoactivation by factor XIa.

Feedback Inhibition
Feedback Augmentation

Adapted from Broze et al., 1992

1.2 Tissue Distribution of TF

The distribution of TF activity in different tissues of the human body was studied by Asrup in 1965. TF activity was found at highest levels in brain, lung and kidney. More recent studies with specific mono- and polyclonal antibodies have allowed the more precise, cell specific localization of TF antigen by immunohistochemical studies, to the vascular adventitia, organ capsules, epithelium of skin and mucosa (Faulk *et al.*, 1990; Fleck *et al.*, 1990; Flossel *et al.*, 1994), Bowman's capsule of glomeruli in the kidney (Flossel *et al.*, 1994) astrocytes in the brain (Flossel *et al.*, 1994; Delzoppo *et al.*, 1992; Eddleston *et al.*, 1993). TF antigen has also been detected in stromal cells of the human endometrium (Lockwood *et al.*, 1993) suggesting that TF serves to promote preimplantation endometrial haemostasis. Unperturbed endothelium and peripheral blood cells did not express TF and fibroblasts showed variable staining patterns. This strategic anatomical localization of TF has led to the hypothesis that TF provides a 'haemostatic envelope', initiating rapid activation of coagulation in the event of vessel damage, whilst at the same time remaining separate from the cells in the circulation to prevent aberrant thrombosis formation. Further support for the hypothesis that TF forms a lining to protect against haemorrhage is the high level of TF antigen in placental tissue, and the observation that oestradiol induced the production of TF mRNA in stromal endometrium in immature rats (Quirk *et al.*, 1995). It is also known that TF is expressed early in human and murine development prior to formation of the circulation (Luther *et al.*, 1996). In addition, during the early stages of TF expression there was no detectable expression of factor VII. This, combined with the recent discovery that TF is a true receptor, (Røttingen *et al.*, 1995) suggests the possibility of other ligands for TF outside the coagulation system. This will be more fully discussed later. TF is thought to be responsible for the continuous generation of small amounts of factors IXa and Xa occurring under basal conditions (Bauer *et al.*, 1990; Bauer *et al.*, 1989a). Such basal activation of coagulation could result from exposure of blood to the trace amounts of TF detectable in normal plasma (Francis *et al.*, 1995) or could occur at extravascular sites where, due to the presence

of factor VII in the interstitial fluids, TF/VII complexes can always be present on cells constitutively expressing TF (Rapaport *et al.*, 1995).

Atherosclerotic plaques were found to contain many cells synthesizing TF including macrophages and intimal cells. The necrotic core of plaques contained cell-free TF protein. These deposits of TF may be involved in the promotion of thrombosis on or in atherosclerotic vessel segments. This topic will be discussed later in the context of TF in disease.

1.3 Physiology and Physical Properties of TF

1.3.1 TF Protein

Tissue factor protein was fully purified from bovine brain in 1981 by Bach *et al.*, and was further characterised simultaneously by Broze and by Nemerson (Broze *et al.*, 1985; Guha *et al.*, 1986) and has recently been assigned the designation CD142. It is a 45kDa, single chain, transmembrane protein, synthesised as a 295 amino acid polypeptide. It consists of two immunoglobulin-like domains associated through an extensive, novel interdomain interface region. In its mature form, TF consists of 263 amino acids organized into a 219 amino acid extracellular domain, which is responsible for binding VII/VIIa, a 23 amino acid transmembrane segment and a 21 amino acid cytoplasmic tail (Morrissey, 1987) (Fig. 1.2) It bears sequence homology to the interferon gamma receptor and so has been classified as a member of the cytokine superfamily of receptors (Bazan, 1990). Unlike other protein cofactors of the clotting cascade, TF is fully functional as a single chain protein and does not undergo proteolytic activation.

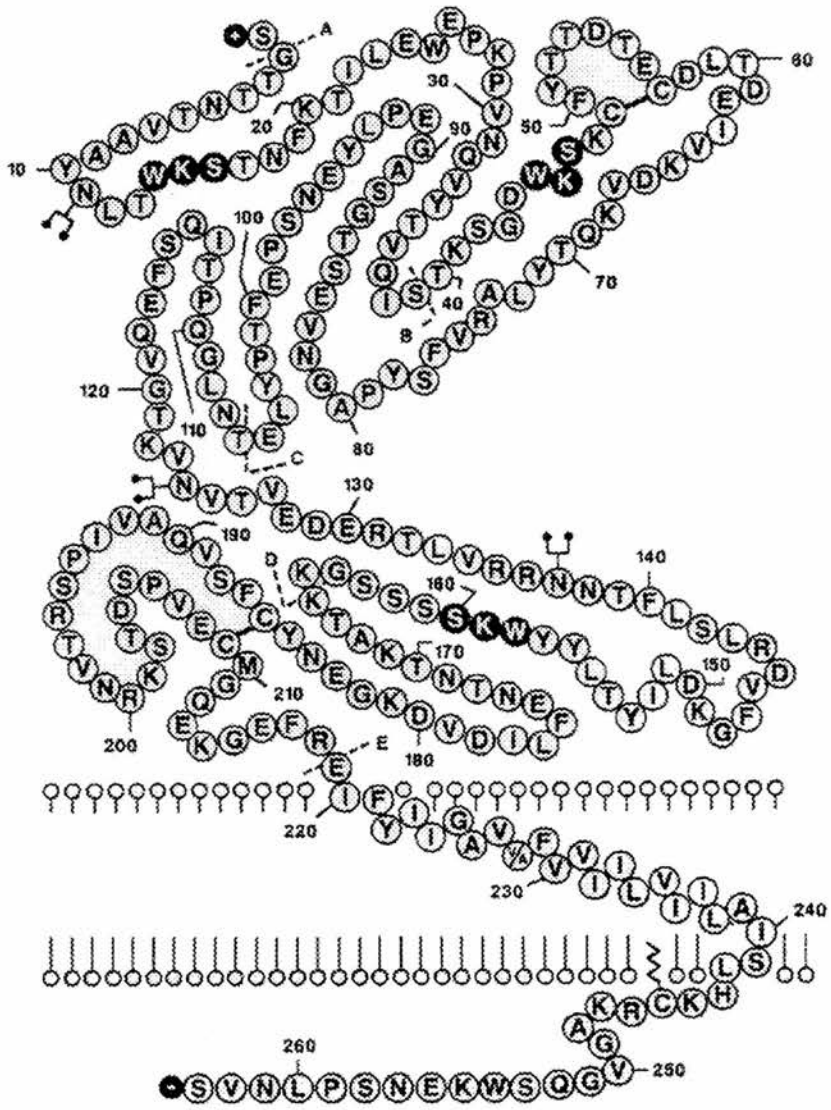


FIGURE 1.2 Structure of the TF Protein. Based on sequence homology with other members of the cytokine/interferon receptor family of proteins, the extracellular region of TF is depicted as two domains that form a V-shaped, ligand-binding trough. The predicted transmembrane domain traverses the plasma membrane on a cell and is followed by a short cytoplasmic tail. The single letter amino acid code is used.

denotes sites of carbohydrate attachment

denotes palmitate or stearate.

The three Trp-Lys-Ser sequences are shown in bold. Site of introns in the TF gene are labelled with capital letters .

Taken from Broze in Haemostasis and Thrombosis, Third Edition, 1994 Volume 1. Eds Bloom, et al. Chapter 15 pp351.

1.3.2 TF as a Receptor

As well as acting as a cofactor for VIIa, TF has now been shown to act as a true receptor by two different groups. To act as a true receptor, a protein must elicit an intracellular signal upon ligand binding. Masuda *et al.*, demonstrated that binding of factor VIIa to cell surface TF on four-day cultured human monocytes could induce transient tyrosyl phosphorylation of intracellular proteins, in particular a 70-kD polypeptide (Masuda *et al.*, 1996). This phosphorylation reached a maximum by 30 seconds with dephosphorylation being complete within a few minutes. This study also reports that TF was co-localised with the 14kDa γ-chain homodimer from the IgEFc receptor. This is a signal transducing element homologous to the zeta chain associated with many other cytokine receptor complexes. Roy *et al.*, 1991 have shown that TF can be cross-linked to both a 70kDa and a 14kDa polypeptide on the cell surface, neither of which have yet been identified. Røttingen *et al.*, 1995 have demonstrated that the binding of factor VIIa can induce transient cytosolic calcium increases in 4 different cell lines: induced HUVECs, constitutive TF producers like Madine-Darby Canine Kidney (MDCK) cells and the human bladder carcinaoma cell line J82, and transfected COS-1 cells. In contrast to the observations of Masuda, Camerer *et al.*, 1996 further noted that this response of MDCK cells was dependent on the proteolytic functionality of factor VIIa and while being inhibited by U73122, a specific inhibitor of phosphoinositol-specific phospholipase C, was not inhibited by a number of tyrosine kinase inhibitors (Camerer *et al.*, 1996a). In this report, the authors also note that the calcium oscillations observed can be triggered by factor Xa as well as VIIa. Both inactivated factor Xa and inactivated factor VIIa show homologous desensitization of receptor function, indicating that the proteins bind to distinct separate receptors on the cell surface. They suggest that the role of TF in the phosphatidyl inositol-specific phospholipase C-mediated Ca^{2+} signalling pathway may be to anchor factor VIIa to the cell surface allowing it to cleave an as yet unidentified protease activated receptor, analagous to the thrombin receptor, rather than itself mediating any transmembrane signal. These studies have been carried out on either cell lines or cultured cells which are likely to behave differently to cells *in vivo* and are

sensitive to culture contaminants. For these reasons it is still not clear as to what class of receptor TF might belong, or indeed if TF actually functions as a signal transduction molecule *in vivo*.

1.3.3 The TF Gene

The human TF gene is 12.4 kbp in length and is located on the short arm of chromosome 1 (Carson *et al.*, 1985; Scarpati *et al.*, 1987; Kao *et al.*, 1988). It is organised into 6 exons which are separated by 5 introns. Exon 1 encodes the N-terminal signal peptide that is removed during processing of the protein. Exons 2-5 encode the extracellular portion of the TF molecule, and exon 6 encodes both the transmembrane and cytoplasmic domains as well as a long 3' untranslated region (Mackman *et al.*, 1989). The 5' untranslated region of the TF transcript is 123 bp in length and the transcription start site is located 26bp downstream from a TATA promoter element. The TF promoter is spanned by a CpG island (Bird, 1986), suggesting that methylation of this region may prevent expression of the TF gene in various cell types including T and B lymphocytes. When serum starved fibroblasts are stimulated with growth factors, TF mRNA expression typically increases and reaches a maximum 1-2 hours after stimulation in the absence of *de novo* protein synthesis (Taby *et al.*, 1996; Bloem et al 1989). This confirms the classification of TF as an 'immediate early' or 'primary response' gene along with c-myc although it is not induced as early as c-fos (Lau *et al.*, 1987).

1.4 Regulation of TF Expression

The TF promoter appears to allow at least three modes of regulation and it is apparent that TF gene expression is regulated differently in different cell types. In most extravascular cell types, the TF gene is constitutively expressed. However, in some of these cell types TF synthesis is turned off by serum/growth factor starvation, and may be induced by reintroduction of these compounds to the medium. In cells such as monocytes, macrophages and endothelial cells the TF gene is not

constitutively expressed but may be induced by a variety of agonists including bacterial lipopolysaccharide (LPS) and Tumour Necrosis Factor-α (TNF-α) (Tables 1-3). Comparison of promoter sequences from human (Mackman *et al.*, 1989) murine (Mackman et al 1992) and porcine (Moll *et al.*, 1995) TF genes reveals conservation of DNA binding sites for several transcription factors that regulate gene expression. These include four Sp1 sites (Kadonaga *et al.*, 1986) Egr-1 (Berg *et al.*, 1992), AP-1 (Lee *et al.*, 1987), and NF-κB (Singh *et al.*, 1988). All these conserved motifs lie within 200bp of the TATA box (Fig 1.3).

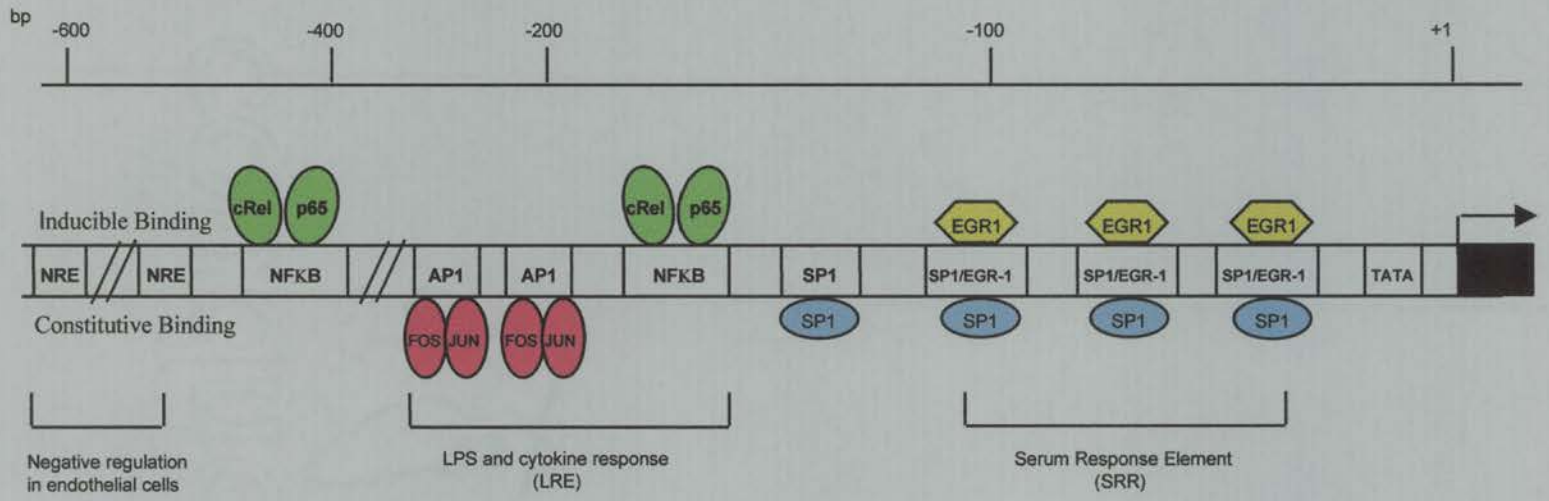


FIGURE 1.3 The Promoter Region of the TF gene.
A summary of constitutive and inducible binding of transcription factors to the promoter of the TF gene. Binding sites for the transcription factors Sp1, Egr-1, c-Rel-p65 and Fos-Jun are indicated. This figure shows the location of the distal enhancer (LRE) which mediates LPS and cytokine induction in monocytes and endothelial cells, and the proximal enhancer (SRR) which mediates serum growth factor induction in epithelial cells.

→ Transcription start site **LPS** Lipopolysaccharide **NRE** Negative Regulatory Element

Taken fromCamerer et al., 1996.

1.4.1 Regulation of Basal Expression (Monocytic Cells, Vascular Endothelial Cells, Vascular Smooth Muscle Cells, Epithelial -Like cells)

To analyze functional expression of the TF promoter, various 5′ flanking regions of the TF gene were cloned upstream of the firefly luciferase reporter gene (Mackman *et al.*, 1990). Initial studies using transiently transfected COS-7 cells, a simian virus 40-transformed monkey kidney cell line which constitutively expresses TF, demonstrated that the TF promoter directs expression of the luciferase reporter gene. Deletion of the promoter from -2106 to -383 did not change the basal transcription of the gene. However, further deletion of the promoter, from -383 to -279 increased the basal transcription, indicating the presence of a negative regulatory element in this region. Subsequent deletion of the region between -278 and -112, which contains both AP-1 sites, the NFκB and a putative Sp1 site decreased basal transcription. However, mutation of the Ap-1 and κB sites indicates that they do not contribute to basal promoter activity. The role of the putative Sp-1 site remains to be determined. These initial studies by Mackman determined that the 'minimal' TF promoter required for basal expression of TF contains three Sp-1 binding sites and spans a region between -111 and +14 bp relative to the start site of transcription. Mutational analysis of this region in HeLa cells suggests that the Sp1 sites are synergistic and that at least two must be retained to maintain a basal level of transcription (Cui *et al.*, 1994).

1.4.2 Induced TF Expression.

Consistent with its potent coagulant activity and immunohistochemical localization, it is apparent that TF is necessarily not normally expressed by intravascular cells. It can, however, be induced *in vitro* in monocytes and endothelial cells by a number of exogenous and physiological stimuli (Tables 1-4). These include endotoxin (LPS) (Rivers *et al.*, 1975), tumour necrosis factor (Bevilacqua *et al.*, 1986), interleukin-1(Nawroth *et al.*, 1986) , immune complexes (Prydz *et al.*, 1979) or phorbol esters (Kornberg *et al.*, 1982). Other reports indicate that gamma interferon (Moon *et al.*,

1988) or granulocyte -macrophage colony stimulating factor (Zuckerman *et al.*, 1989) can act synergistically with LPS to induce macrophage associated TF.

1.4.2.1 Serum Growth Factor and Phorbol Ester (PMA) Induction of TF Gene Expression.

Stimulation of quiescent murine and human fibroblasts with serum or mitogens rapidly (1-2 hours) induced TF mRNA without the requirement for *de novo* protein synthesis (Lau *et al.*, 1987; Hartzell *et al.*, 1989; Bloem *et al.*, 1989; Ranganathan *et al.*, 1991). In quiescent rat vascular smooth muscle cells (Taubman *et al.*, 1993) and in human epithelial cells (Cui *et al.*, 1994), TF synthesis was induced by PMA as well as by serum growth factors. Induction both by serum and by PMA requires calcium, whereas only PMA induction requires protein kinase C (Taubman *et al.*, 1993; Cui *et al.*, 1994), suggesting that serum and PMA induction of TF expression is mediated by different signalling pathways. Mackman has suggested that the serum response region (SRR) of the TF gene is located between residues -111 to +14 on the TF promoter and is unrelated to the c-fos serum response element. Mutational analysis of this region in HeLa cells, demonstrated a redundancy in this element: deletion of individual Sp-1 sites failed to inhibit induction indicting that no individual element was absolutely required for the induction of TF expression, rather several distinct DNA elements may act in concert to regulate serum and PMA induction. (Cui *et al.*, 1994). Gel shift mobility assays were subsequently used to analyze the binding of nuclear proteins to radiolabelled oligonucleotides spanning the SRR between -111 and +14 bp (Cui *et al.*, 1994). These studies revealed constitutive binding of Sp-1 to all three Sp-1 sites in the SRR. In addition, serum or PMA stimulation of HeLa cells induced *de novo* expression of Egr-1 that bound to three sites that overlapped the three Sp-1 sites. Erg-1 is an immediate early gene that is transiently expressed in fibroblasts and epithelial cells in response to stimulation with serum growth factors (Madden *et al.*, 1993). Interestingly, induction of Erg-1 mRNA and protein precedes induction of TF mRNA in serum stimulated fibroblasts (Hartzell *et al.*, 1989). Recent studies in HeLa cells using plasmids containing mutations in both the Sp-1 and the Egr-1 sites have

indicated that both the Sp-1 and the Egr-1 recognition sites are involved in the regulation of the serum induction of the TF gene (Cui *et al.*, 1996).

1.4.2.2 LPS and Cytokine Monocytic Cell TF Induction

Recent observations have indicated that thrombosis may be triggered by the aberrant expression of TF on the surface of activated peripheral blood monocytes (Barstad *et al.*, 1995). Inhibition of protein or RNA synthesis inhibits the expression of TF procoagulant activity in stimulated monocytes suggesting that there is not an intracellular pool of presynthesised TF and that *de novo* synthesis is required for monocytes to express procoagulant activity (Gregory *et al.*, 1989; Crossman *et al.*, 1990). *In vitro*, bacterial LPS, cytokines and PMA are among the agonists that induce TF expression in monocytes. TF synthesis is generally induced by agents which increase the levels of cytosolic calcium, and suppressed by agents which increase the levels of cyclic AMP (Lyberg *et al.*, 1983; Prydz *et al.*, 1980). There are at least three cell surface receptors for LPS, CD14 (Wright *et al.*, 1990), CD11/CD18 (Wright *et al.*, 1986) and a 80kDa protein (Bright *et al.*, 1990), and occupancy and involvement of these is possibly dependent on the amount of LPS applied (Ternisien *et al.*, 1995). Difficulties in transfecting primary monocytes have precluded the direct analysis of the TF promoter in these cells. Studies by Mackman *et al.*, have focused on a human monocytic cell line, THP-1, which is derived from an acute human monocytic leukaemia and which induces TF gene expression in a manner similar to peripheral blood monocytes, to investigate regulation of the human TF gene (Brand *et al.*, 1991). The region of the TF promoter responsible for activation of TF synthesis by LPS and cytokines appears to be distinct from the serum response region. Deletion analysis of the TF promoter in human monocytic cells allowed Mackman *et al.*, to restrict the LPS response to a distal enhancer (LRE), a 56 bp region from -227 to -172 containing the two AP-1 like motifs and one NF-κB like motif (Mackman *et al.*, 1991). Recent functional studies by Mackman *et al.*, using THP-1 cells show that LPS induction of the LRE is abolished by mutation of either of the AP-1 sites or the κB site (Oeth *et al.*, 1994; Parry *et al.*, 1995). Upon stimulation with LPS through

the CD14 receptor, the TF promoter binds c-Jun, Fos being constitutively bound (Groupp *et al.*, 1996) suggesting that the initiation complex involves an interaction between c-Rel/p65 on the NFκB site and c-Jun/Fos on the AP1 site (Mackman et al, 1995; Stein *et al.*, 1993). Prior to LPS stimulation, the c-Rel-p65 complex is retained in the cytosol in complex with the inhibitor IκBα (Oeth *et al.*, 1994; Parry *et al.*, 1995). Activation of the complex requires dissociation and proteolytic degradation of the inhibitor before nuclear translocation of the complex (Mackman *et al.*, 1994; Henkel *et al.*, 1993). MAD3, an IκB protein that has been shown to be involved in downregulation of NFκB translocation to the nucleus (Zabel *et al.*, 1993) is phosphorylated in THP-1 cells upon LPS stimulation (Cordle *et al.*, 1993). This suggests that upregulation of TF expression upon LPS stimulation may be due to phosphorylation and degradation of the inhibitor IκB (Finco *et al.*, 1995), allowing the translocation of the c-Rel-p65 complex to the nucleus and subsequent binding to the TF promoter. A number of studies have suggested that LPS can induce NFκB activation via a number of different mechanisms. In CHO cells (which do not normally respond to LPS) transfected with a CD14 expressing plasmid and RAW 264.7 cells (Delude *et al.*, 1994) NFκB nuclear translocation does not require tyrosine kinase activity. However, NFκB activation by LPS was inhibited in c6 glicoma cells by incubation with the tyrosine kinase inhibitor herbimycin A (Nishiya *et al.*, 1995).

1.4.2.3 Endothelial Cell TF Induction

Vascular endothelial cells have also been shown to express TF in cell culture when stimulated by LPSs (Colluci *et al.*, 1983), thrombin (Brox *et al.*, 1984), IL-1 (Bevilacqua et al,. 1986) and TNF-α (Bevilacqua *et al.*, 1986). Cytokine and LPS induction of the human and porcine TF promoters transfected into human, porcine and bovine endothelial cells is mediated by the binding of transcription factors to the two AP-1 sites and the NFκB site within the LRE (Bierhaus *et al.*, 1995; Bierhaus *et al.*, 1995; Moll *et al.*, 1995; Parry *et al.*, 1995) in a manner similar to human monocytic cells. In addition, over expression of the inhibitor IκBα dramatically reduces TF expression in stimulated porcine endothelial cells (Wrighton *et al.*, 1996),

confirming a role for c-Rel-p65 in inducible TF expression in these cells. Mackman *et al.*, have also shown that PMA induction of the TF promoter in human umbilical vein endothelial cells (HUVEC) is mediated by the LRE and a second PMA response element containing three Egr-1 sites (Parry *et al.*, 1995). Unpublished data by Mackman *et al.*, have shown that PMA induces Egr-1 in HUVECs suggesting that Egr-1 may contribute to the induction of TF gene expression in endothelial cells as has been shown in monocytic cells. However, it is widely believed that TF induction in endothelial cells is an *in vitro* phenomenon as expression of TF in the endothelium has been observed only rarely *in vivo*. Saphenous veins and internal mammary arteries from coronary bypass surgery and atherscleroticplaques from carotid endarterectomy specimens failed to show TF expression (Wilcox *et al.*, 1989). Furthermore, immunocytochemical studies of *E. coli* induced DIC in baboons revealed endothelial TF expression associated only with the endothelium in the splenic microvasculature (Drake *et al.*, 1993). TF has, however, been found in subendothelium in ballooned human and rabbit arteries (Weiss *et al.*, 1989), and after *ex vivo* TNFα stimulation of segments of bovine aorta (Ryan *et al.*, 1992) but not after *ex vivo* LPS or thrombin stimulation of isolated intact human saphenous veins (Solberg *et al.*, 1990). In humans, TF is expressed in vascular endothelial cells within tumours of patients with invasive breast cancer (Contrino *et al.*, 1996). Limited activity seen in some studies may be caused by the presence of TFPI. Lack of immunological detection may be caused by sensitivity limitations, as the level of induced TF expression in endothelial cells is lower than the constitutive expression in other cell types (Diquelou *et al.*, 1995). It is also possible that there is heterogenous expression of TF in endothelial cells (Kirchhofer *et al.*, 1994; Mackman *et al.*, 1995) or that repressor systems might limit the expression of TF in endothelial cells *in vivo*. Østerud, 1995b has also proposed that the endothelium is not procoagulant in itself but it may support reactions that enhance TF generation in the vascular system, such as the binding and activation of neutrophils and monocytes and their interactions with platelets. Alternatively, it may still be possible that TF is expressed in endothelial cells *in vivo*, but that the induction is more tightly controlled or more difficult to detect than in the cell culture situation.

Inducing Agent	Monocyte Lineage	Endothelial Cell Lineage
Bacterial Lipopolysaccharide	Rivers *et al.*, 1975	Lyberg *et al.*, 1981
Rickettsia rikettsii		Sporn *et al.*, 1994
Rickettsia conorrii		Teysseire *et al.*, 1992
N.Meningitdis	Østerud *et al.*, 1983	
M. leprae/M. bovis	Lyberg *et al.*, 1982	
Serum from P.falciparum malaria patients		Hemmer *et al.*, 1991
Antiphospholipid sera		Branch & Rodgers, 1993
Monoclonal anticardiolipin antibodies	Kornberg *et al.*, 1994	
Mouse hepatitis virus	Levy *et al.*, 1981	
Lectins	Lyberg *et al.*, 1980	
Phorbol ester	Lyberg *et al.*, 1981	Lyberg *et al.*, 1981
Calcium ionophores	Prydz *et al.*, 1990	
Diacylglycerol		Petterson *et al.*, 199
Amines	Dean *et al.*, 1983	

TABLE 1.1 Induction of TF Synthesis by Exogenous Agents.
Adapted from Camerer *et al.*, 1996

Inducing Agent	Monocyte Lineage	Endothelial Cell Lineage
Thrombin		Brox *et al.*, 1984, Gladal *et al.*, 1985
Interleukin 1α and β	Bevilacqua *et al.*, 1986, Carlsen et al 1988	Carlsen et al 1988
Interleukin 2		Carlsen et al 1988
Tumour Necrosis Factor α		Bevilacqua *et al.*, 1986;Nawroth *et al.*, 1986; Carlsen et al 1988
VEGF	Clauss *et al.*, 1990	Clauss *et al.*, 1990
P-selectin	Celi et al 1994	
Immune complexes	Prydz *et al.*, 1979	
Heat-aggregated IgG		Tannenbaum *et al.*, 1986
Complement factors	Prydz *et al.*, 1977	
Histamine		Galdal *et al.*, 1984
Adrenaline		Galdal *et al.*, 1984
C-reactive protein	Cermak *et al.*, 1993	
Chemically modified LDL	Schuff-Werner *et al.*, 1989	
Minimally oxidized LDL		Drake *et al.*, 1991
Exogenous free cholesterol	Lesnik *et al.*, 1992	
Procoagulant albumin	Faucette *et al.*, 1992	Faucette *et al.*, 1992
Tuftsin	Kornberg *et al.*, 1990	
Oxidizing conditions	Crutchley *et al.*, 1995	
Monocyte chemoattractant protein	Siegbahn *et al.*, 1995	
Platelet-derived growth factor BB	Siegbahn *et al.*, 1995	

TABLE 1.2. Induction of TF Synthesis by Physiological Agents

Adapted from Camerer *et al.*, 1996

Inducing Condition	Monocyte Lineage	Endothelial Cell Lineage
Allogenic lymphocytes	Carlsen *et al.*, 1989	Lyberg *et al.*, 1983;Carlsen *et al.*, 1989
Monocyte adherence activated EC	Lo *et al.*, 1995	
Platelet adherence	Niemetz & Marcus 1974	Johnsen *et al.*, 1983
Hypoxia		Gertler *et al.*, 1991
Phagocytosis		Dean *et al.*, 1983

TABLE 1.3. Induction of TF Synthesis by Cell Interaction and Other Conditions
Adapted from Camerer *et al.*, 1996

Inducing Agent	Epithelial Lineage	Vascular Smooth Muscle Cells	Fibroblast Lineage
Serum	Cui *et al.*, 1994	Taubman *et al.*, 1993	Bloem *et al.*, 1989
Platelet derived growth factor		Taubman *et al.*, 1993	Hartzell *et al.*, 1989
Fibroblast growth factor			Hartzell *et al.*, 1989
Epidermal growth factor		Taubman *et al.*, 1993	Ranganathan *et al.*, 1991
Transforming growth factor β1			Ranganathan *et al.*, 1991
Insulin			Ranganathan *et al.*, 1991
Alpha-thrombin		Taubman *et al.*, 1993	
Phorbol ester	Cui *et al.*, 1994	Taubman *et al.*, 1993	
Angiotensin II		Taubman *et al.*, 1993	

TABLE 1.4. Stimulation of TF synthesis in Serum Starved Cells Normally Expressing TF.

Adapted from Camerer *et al.*, 1996

1.4.3 Control of TF Synthesis

It has been reported that TF procoagulant activity is greater in lysed cells than in intact cells (Carson *et al.*, 1986; Maynard *et al.*, 1975). However, it has been variously shown that TF protein is entirely expressed on the cell surface of most cells. Therefore, it appears that the catalytic activity of the TF/VIIa complex on most intact cells is markedly less than that in cell lysates suggesting that alterations in membrane lipids may be a means of regulating TF procoagulant activity. Treatment of cells with calcium or an ionophore can mimic the TF increase seen with cell disruption, suggesting that there may be a calcium associated physiological control of membrane activity at this level (Bach *et al.*, 1990). The complex pattern of TF expression in different tissues and cell types suggests that regulation of TF procoagulant activity involves different regulatory mechanisms. In monocytes, LPS treatment induces transient *de novo* transcription of the TF gene resulting in a transient increase in TF activity (Gregory *et al.*, 1989; Mackman *et al.*, 1990). Therefore, it is apparent that the primary level of regulation in the monocyte is at the level of transcription. However, it has also been shown that TF can be regulated by post transcriptional mechanisms. A report by Brand et al.1991 has demonstrated that TF mRNA stability changes after LPS stimulation. One hour after LPS treatment, the half-life of TF mRNA was greater than 120 minutes, whereas 2 hours after treatment, the half life declined to 25 minutes. It has also been reported that high concentrations of PMA can induce a rapid decrease in membrane associated TF in peripheral blood monocytes, which suggests that TF can be modulated at the level of the plasma membrane (Brozna *et al.*, 1988). From these experiments it is apparent that TF activity is regulated by several different mechanisms that modulate turnover of membrane associated TF, stability of TF mRNA, TF gene transcription or translation of TF mRNA.

1.4.3 1 Posttranscriptional Control of TF Expression

The first level of control after transcription lies in the stabilization/destabilization of the primary transcript. Transcription of the TF gene begins within 15 minutes of treatment with LPS and reaches a maximum level after 1 hour. Tissue factor mRNA transcripts accumulate to maximum levels within 4 hours and then decrease rapidly (Gregory *et al.*, 1989). In monocytes, the transient increase in TF mRNA levels correlates with a transient increase in TF procoagulant activity which is maximal 4-6 hours after LPS treatment. It is, therefore apparent that expression of TF procoagulant activity is dependent on continuous TF mRNA synthesis, since when TF mRNA synthesis ceases, TF procoagulant activity declines. The major TF transcript is 2.2 kb in length, but in several cell types and tissue from placenta, a small proportion of longer transcripts (3.1 - 3.4 kb) is seen (Scarpati *et al.*, 1987). In LPS stimulated monocytes, the longer transcript arises from an alternative splicing including 955 bp on intron 1 (Vanderlogt *et al.*, 1992; Brand *et al.*, 1991). As intron 1 contains an inframe stop codon, no protein is produced from this transcript. During the stimulation of THP-1 cells, only the smaller (2.2-kb) transcript was detected initially, but after 4 hours the larger 3.4 kb transcript was present and appeared to be more stable (Brand *et al.*, 1991).

1.4.3.2 Posttranslational Control of TF Expression

After production of the mature protein, the next step in the regulation of TF expression is its cellular localisation. In order to serve as a receptor/cofactor for factor VII, TF must be present on the cell surface in a suitable configuration to initiate coagulation. It has been reported that not all of the TF/VII complexes on the cell surface are capable of supporting coagulation (Le *et al.*, 1992) in that TF may be present but not functional. TF may be active but not functional if expressed in a region where it is unavailable to the plasma clotting factors except on cell lysis. TF activity increases dramatically following cell lysis, the surface activity being typically 20% of the activity observed after lysis (Drake *et al.*, 1989; Le *et al.*, 1992; Rao *et*

al., 1992). Binding of neutralizing monoclonal antibodies to intact monocytes resulted in more than 90% inhibition of the TF activity in lysed cells, suggesting that TF is predominantly surface expressed (Drake *et al.*, 1989). This was also observed in the human ovarian carcinoma cell line OC-2008 (Rao *et al.*, 1992). In contrast to these results, TF antigen has been found in subendothelial vesicles of HUVECs and not on the apical surface (Ryan *et al.*, 1992). Tissue factor activity is thought to be greater in lysed cells due to a component of the plasma membrane which is unavailable on the cell surface but exposed on cell disruption. This component is thought to be phosphatidylserine (PS), which is crucial for TF activity (Bach *et al.*, 1986; Bjørklid & Storm, 1977) In fibroblasts calcium ionophores have been shown to rapidly increase surface TF activity without changing antigen levels (Bach *et al.*, 1990). Conversely, thrombin stimulation, whilst increasing cytosolic calcium, did not produce the same effect as ionophores (Carson *et al.*, 1994) These studies concluded that there is a limiting amount of PS in the outer leaflet of the plasma membrane, and that cell lysis increased TF activity by increasing the amount of PS available to the TF/VIIa complex. They also suggest that redistribution or 'flip-flopping' of membrane PS may be a way of modulating TF activity or by cell lysis to modulate TF activity. Recently, two groups have reported that TF is localized to specific regions of the plasma membrane (Sevinsky *et al.*, 1996; Mulder *et al.*, 1996). Sevinsky *et al.*, 1996 have shown that subsequent to TF/factorVIIa complexes forming on the endothelial cell surface, they are actively transported to membrane domains called caveolae. Futhermore, Mulder *et al.*, have demonstrated that on smooth muscle cells TF is localized to caveolae without the formation of enzyme/substrate complexes. It has been reported that caveloae are deficient in anionic phospholipid binding sites (Simionecu *et al.*, 1981a; Simionecu *et al.*, 1981b) but are rich in signalling molecules including 1,4,5,-triphosphosphate receptors and protein kinase C (Smart *et al.*, 1995). Therefore, TF in caveloae could be speculated to be either a latent pool of procoagulant activity (Mulder *et al.*, 1996) or a downregulation of function (Sevinsky *et al.*, 1996). Another mode of regulation of TF activity is the surface turnover of the protein. The activity of transmembrane proteins is often controlled by internalization, recycling or exocytosis from intracellular pools. Zioncheck *et al.*, 1992, have

reported that although there are no endocytosis motifs in the cytoplasmic domain of the TF molecule there is a conserved serine/threonine phosphorylation site which might be a good candidate for control of membrane localization. This group also reported that staurosporine inhibited this phosphorylation. As staurosporine has also been reported to block downregulation of TF (Brozna *et al.*, 1994) it might be concluded that this phosphorylation is related to stabilization of the TF molecule on the cell surface.

1.5 Tissue Factor in Disease

In recent years numerous studies have focused on the *in vivo* expression of TF in health and disease. The selective perivascular, cell-specific distribution of TF and the lethal effects of TF knockouts have added support to the view that TF plays a pivotal role in the initiation of blood coagulation during physiological haemostasis. Inappropriate *in vivo* expression of TF, particularly by intravascular cells that do not express TF under normal circumstances (monocytes, macrophages and endothelial cells), has been widely documented and is accepted to be responsible for aberrant clot formation in a variety of pathological conditions including sepsis associated disseminated intravascular coagulation (DIC) and thromboembolic disease. In malignancy, *in vivo* expression of TF by tumour cells and/or by host cells has been implicated not only in intratumoral and systemic activation of blood coagulation but also in tumour growth and metastasis. Some of these disease states will be discussed in detail below.

1.5.1 Endotoxaemia/DIC

Disseminated intravascular coagulation (DIC) arises due to inappropriate and excessive activation of the coagulation process. Increased fibrin formation may lead to diffuse microvascular thrombosis resulting in impairment of bloodflow, ischaemia, multiple organ failure and subsequent death. Many different underlying diseases are associated with DIC, including septicaemia, obstetric complications, severe trauma

and malignancy (Bell *et al.*, 1994). Exposure of TF to the peripheral bloodstream is believed to be a common factor in the initiation of aberrant coagulation in DIC. This may be due to direct exposure of preformed TF (during obstetric accidents or severe trauma) or to induction of TF expression on the surface of activated monocytes such as in sepsis (Brozna *et al.*, 1990). In cancer, tumour cells may synthesize excessive amounts of TF which may result in intravascular coagulation (Edwards *et al.*, 1981). Infusion of recombinant TF initiates DIC in rabbits, strongly suggesting that TF is able to cause the disease (Warr *et al.*, 1990). Disseminated intravascular coagulation following gram negative septicaemia is the clearest model for the pathophysiological significance of the aberrant expression of TF in cells in contact with the blood. To establish the role of TF in DIC, several animal models of lethal intravenous infusion of bacteria have been utilized in rabbits, mice, baboons and chimpanzees. In rabbits, mononuclear macrophages from various origins (blood, peritoneal cavity, pulmonary alveoli, bone marrow and spleen) and aortic endothelium obtained after endotoxin infusion express strong TF activity (Semeraro *et al.*, 1981; Rothberger *et al.*, 1983; Montemurro *et al.*, 1985; Semeraro *et al.*, 1993). Furthermore, in mice treated with endotoxin, TF mRNA was elevated in lung and kidney. In situ hybridization studies further localized the mRNA to alveolar type II cells in the lung and tubular epithelial cells in the kidney, whereas endothelial cells were negative (Mackman *et al.*, 1995; Mackman *et al.*, 1993b). In baboons with *E. coli* septic shock, TF antigen was detected in the splenic endothelial cells and macrophages as well as in lung alveolar epithelial cells and glomerular epithelial cells (Drake *et al.*, 1993). These experiments coupled with the earlier observations that a normal number of leukocytes is required for endotoxin induced DIC to occur, and that passive transfer of TF expressing leukocyes into normal animals causes DIC (Muller-Berghaus *et al.*, 1978) support the concept that mononuclear phagocytes play a key role in endotoxin induced DIC. In addition, monocytes from endotoxin resistant animals show a marked reduction in TF expression when treated with endotoxin (Semeraro *et al.*, 1992). Furthermore, both TF neutralizing antibodies (Warr *et al.*, 1990; Taylor *et al.*, 1991; Levi *et al.*, 1994), factor VII/VIIa neutralizing antibodies (Biemond *et al.*, 1995), TFPI (Day *et al.*, 1990; Creasey *et al.*, 1993) and more recently active-site inhibited factor VIIa (Taylor

et al., 1997), reverse the lethality of endotoxin infusion in primate models, indicating that TF dependent initiation of coagulation is responsible for the diseased state. Moreover, depletion of TFPI in a rabbit model sensitized the animals to DIC (Sandset *et al.*, 1991a; Sandset *et al.*, 1991b).

Activation of coagulation is almost invariably observed in human sepsis However, the pathophysiological mechanisms involved have been more difficult to elucidate than in animals. As a result, human studies have been restricted to analysis of plasma or circulating monocytes/macrophages. In severe infectious diseases such as meningococcal infection and bacterial peritonitis peripheral blood monocytes exhibited a significant increase in TF activity (Østerud *et al.*, 1983; Almdahl *et al.*, 1987). Interestingly, the highest levels of cellular TF were seen in patients with lethal outcome suggesting a prognostic value for this measurement. A substantial increase in TF activity has also been reported in peritoneal macrophages from patients with peritonitis (Almdahl *et al.*, 1987), and in the bronchiolar lavage fluid of patients with adult respiratory distress syndrome secondary to sepsis (Idell *et al.*, 1989). Tissue factor can also be found in plasma from patients with DIC (Takahashi *et al.*, 1994; Wada *et al.*, 1994). Taken together, these studies demonstrate an important role for TF in the initiation of coagulation in DIC.

It is of note that studies investigating LPS induction of clotting activity most often utilize *E. coli* LPS. It is clear, however, that LPSs are a complex set of molecular species the activities and functionality of which vary greatly and are dependent on their structures and bacterial source. One of the most common causes of human sepsis is bacterial infection following peritonitis. It is known that bacteria from the *Bacteriodes* species outnumber *E. coli* in the gut by 1000 fold (Delahooke *et al.*, 1995), and so it is likely that these bacteria will be the major cause of infection in disease complications involving gut rupture. It is, therefore, important to consider the different TF inducing potential of these different bacterial LPSs. This is addressed further in chapter 7.

1.5.2 Athersclerosis and Arterial Thrombosis

Thrombosis is linked to the development and progression of atherosclerosis. Acute thrombosis can occur spontaneously leading to arterial blockage resulting in myocardial infarction, unstable angina, stroke and sudden death (Fuster *et al.*, 1992). Recent studies suggest that the accumulation of TF in atherosclerotic plaques plays a major role in determining plaque thrombogenicity. TF is also rapidly induced in the vessel wall as a consequence of acute arterial injury. Both phenomena may be important in the thrombotic complications of atherosclerotic heart disease. Monocytes are instrumental in the development of athersclerotic lesions. When differentiated to macrophages in the vessel wall, they are scavengers of oxidised LDL. Thus they are transformed into foam cells, an early event in the atherogenic process. Studies in cell culture systems have demonstrated that most key cellular elements within an atherosclerotic plaque, including endothelial cells, mononuclear macrophages and smooth muscle cells, are able to synthesize TF in response to a number of atherogenic molecules including cytokines, growth factors, modified low density lipoproteins and cell interactions. Furthermore, in perfusion models of thrombogenesis, TF-expressing monocytes and endothelial cells, when exposed to nonanticoagulated blood under flow conditions, could elicit fibrin deposition and thrombus formation (Clozel *et al.*, 1989; Barstad *et al.*, 1995). These studies prompted the investigation of *in vivo* TF expression in these cells and their role in arterial thrombosis both in animals and in humans. In rabbit atherosclerotic lesions, induced by cholesterol rich diets, TF antigen and activity were detected in foamy and non-foamy macrophages and smooth muscle cells but not by endothelial cells (Kato *et al.*, 1996). In rabbits fed an atherogenic diet for six weeks, spleen mononuclear phagocytes expressed increased basal TF activity, and exhibited an enhanced procoagulant response to endotoxin both *in vitro* and *in vivo* (Semeraro *et al.*, 1990). Studies using a rat balloon injury model, Marmur *et al.*, 1993, demonstrated a rapid induction of TF mRNA and activity in medial smooth muscle cells of aorta in response to balloon dilatation. In addition, Speidel *et al.*, 1995, observed that TF caused prolonged procoagulant activity associated with the luminal surface of balloon injured

rat aortas. Several studies have also examined the expression of TF in human atherosclerosis. Wilcox *et al.*, 1989 first reported that, in atherosclerotic plaques from patients undergoing carotid endartectomy, TF mRNA and protein were expressed in macrophage foam cells, monocytes, smooth muscle cells but not in endothelial cells. TF protein was also present in the extracellular matrix of the necrotic core, possibly shed from the surface of adjacent TF expressing cells. Consistent with these findings, macrophages isolated from carotid atherectomy patients expressed markedly increased TF activity (Tipping *et al.*, 1989). Patients with a history of recent embolic complications of carotid vascular disease, had TF levels far exceeding those of plaque macrophages from asymptomatic patients. Annex *et al.*, 1995, first demonstrated the presence of TF antigen in coronary lesions using immunohistochemical staining of coronary atherectomy specimens. The detection rate was higher in patients with unstable coronary syndromes than in patients with stable disease. In contrast, Marmur *et al.*, 1996 used a specific functional assay and Thiruvikraman *et al.*, 1996 a novel method based on the binding of labelled factors VIIa and X to show that TF was present in variable amounts in virtually all of the plaques tested, but was not correlated to clinical severity. Within the plaque, TF was particularly abundant in the relatively acellular lipid-rich core and was also located in the surrounding smooth muscle cells. In contrast to other studies, the endothelium overlying the plaques was found to be often positive for TF (Thiruvikraman *et al.*, 1996). Taken together, these studies strongly suggest that the prognosis for plaque rupture is determined by expressed TF which causes rapid thrombus formation. The importance of TF in this regard has been further demonstrated by the preventative effect of TFPI on arterial reocclusion (Haskel *et al.*, 1991). An increased capacity to produce TF has been reported also in circulating monocytes from patients with coronary disease and in particular those with active unstable angina (Neri *et al.*, 1992; Jude *et al.*, 1994; Leatham *et al.*, 1995) possibly reflecting a specific inflammatory process, that leads to increased TF expression, thrombin generation and thrombus formation and may contribute to plaque instability. Most recently, it has been shown that extracts from human atheromatous plaques express constitutive TF activity and also induce TF activity in monocytes (Muhlfelder *et al.*, 1999).

1.5.3 Venous Thrombosis

Although it is known that activation of blood coagulation, in combination with blood stasis is an essential pathogenic factor in venous thrombosis the precise initiation event is still unclear (Thomas, 1994). One possibility is that activation of coagulation is caused by the release of TF from injured tissues into the bloodstream. Alternatively, thrombus formation in areas of stasis could be initiated by the exposure of blood to locally available TF, possibly as a result of vessel wall damage, endothelial cell activation or recruitment of activated monocytes (Thomas, 1994). Vessel wall lesion, exposing preformed TF, does not appear to play a major role except in the case of severe vascular trauma. In a rabbit jugular vein stasis model, the injection of TF-expressing blood mononuclear cells induced thrombus formation more quickly than a rabbit thromboplastin preparation (Scagnol *et al.*, 1991). Moreover, the administration of low doses of endotoxin 4 hours before stasis potentiated venous thrombosis in normal but not leukopenic rabbits (Bernat *et al.*, 1994). Thus, monocyte-initiated coagulation could contribute to thrombus formation in areas of blood stasis. In humans, evidence linking *in vivo* expression of monocytes TF to the pathogenesis of venous thrombosis remains inconclusive. An augmented monocyte TF activity has been reported in conditions known to be associated with venous thromboembolism, including major surgery and trauma (Miller *et al.*, 1981; Blakowski *et al.*, 1986; Ollivier *et al.*, 1989).

1.5.4 Malignancy

The tendency to thrombotic complications in various types of malignant disease is thought to be partly due to a constitutive or induced expression of TF. Several tumours express TF constitutively (Callander *et al.*, 1992a; Sturm *et al.*, 1992; Zhang *et al.*, 1994), and there is also strong evidence to suggest that local activation of coagulation and fibrin deposition occurs at sites of tumour growth (Dvorak *et al.*, 1994). Several *in vivo* studies on the expression of TF in human malignancy have shown that in both haematological disease (Tanaka *et al.*, 1993) and in solid tumours

(Callander *et al.*, 1992; Costantini *et al.*, 1993; Hamada *et al.*, 1996), TF antigen, activity and mRNA have been detected. As many tumour cells express TF, systemic haemostatic abnormalities may result from malignant cell entry into the circulation during the course of metastasis or from TF expressing membrane vesicles shed from tumour cells. Alternatively, coagulation may be activated by release of tumour or host cell derived mediators that stimulate monocytes and endothelial cells to express TF. The fact that patients with malignant disease show increased circulating monocyte TF which often correlates with markers of activated coagulation (Morgan *et al.*, 1988; Semeraro *et al.*, 1994; Montemurro *et al.*, 1995) lends support to this view. In addition, high plasma and urinary levels of TF have been frequently reported in cancer patients (Kakkar *et al.*, 1995; Levi *et al.*, 1993). Recent studies have also demonstrated the involvement of TF in tumour angiogenesis. In a study using meth-A-sarcoma cells which had been induced to either over or under express TF, it was shown that TF may contribute to the angiogenic properties of tumour cells by altering the production of growth regulatory molecules. Furthermore, TF has been localized to the vascular endothelial cells within invasive breast cancer tumours (Contrino *et al.*, 1996).

1.5.5 Surgical Trauma (Cardiopulmonary Bypass)

During cardiopulmonary bypass (CPB) surgery there is an extensive activation of the extrinsic coagulation pathway. Experimental observations have attributed this to the widespread circulation, during cardiac surgery, of inflammatory mediators such as tumour necrosis factor and interleukin-1 as well as LPS which can induce the expression of TF on monocytes and endothelial cells. These observations are supported by the fact that many of the features of CPB-associated coagulopathy can be induced in animals by infusion of LPS or cytokines (Nawroth *et al.*, 1986; Bauer *et al.*, 1989b). During CPB there is the potential for massive widespread TF expression throughout the entire extravascular space due to the systemic exposure of monocytes and endothelial cells to inflammatory mediators. If left unchecked, this would result in intravascular coagulation and widespread fibrin deposition in the microvascular

circulation. There are a variety of therapies directed at attenuating the effects of widespread thrombin generation during CPB. Systemic heparinization is intended to stop the progression of the coagulation cascade. However, thrombin generation increases after the neutralization of heparin with protamine sulphate, and continues to be elevated significantly 24 hours post-operatively (Kestin *et al.*, 1993). In addition, heparin does not directly inhibit the formation of thrombin and, therefore, cannot prevent widespread microvascular thrombosis. Other agents such as serine protease inhibitors and platelet inhibitors have also been used but like heparin, they focus on inhibiting coagulation distal in the cascade, once endothelial cells have been activated, TF has been expressed, and widespread thrombin generation has occurred.

1.6 Therapeutic Potential of TF Inhibition

The predictable way in which TF is expressed in a variety of disease states where aberrant coagulation plays a role, suggests that strategies aimed at inhibition of TF may be useful as novel anti-thromboembolic therapies. Studies have been performed in animal models that demonstrate the importance of TF in the initiation and progression of thrombotic complications and illustrate the protective effect of TF inhibition at the protein, intracellular and molecular level in these conditions.

1.6.1 Monoclonal Antibodies to TF

The infusion of monoclonal antibodies to TF has been shown to be beneficial in a number of animal models of DIC and arterial thrombosis. Intravenous infusion of *E. coli* at a lethal dose into a baboon is associated with coagulopathy, vasomotor collapse, organ failure and death (Coalson *et al.*, 1979). Injection of either the IgG or Fab of anti-TF monoclonal antibody TF9-5B7 protected baboons from the effects of this lethal dose of *E. coli*. Infusion of small amounts of this monoclonal antibody before *E. coli* challenge attenuated the septic coagulopathy, cardiovascular collapse and cell injury involved in DIC resulting in 100% survival rate (Taylor *et al.*, 1991). In another study injection of a polyclonal anti-TF antibody into mice 2 hours before

intraperitoneal injection of LPS, resulted in a reduction in the mortality rate compared to saline infused controls (Dackiw *et al.*, 1996). Inhibition of TF using monoclonal antibodies has also been shown to prevent acute coronary and vascular thrombosis. Three animal studies have addressed the posibility of inhibiting vascular thrombosis by the use of anti-TF antibodies. Jang *et al.*, 1992, exposed adventitial TF to the bloodstream in an eversion graft model of rabbit femoral artery. A 2 hour infusion of a neutralizing anti-TF monoclonal antibody prevented thrombosis in 80% of the animals. Furthermore, in a rabbit model of carotid artery thrombosis, administration of a monoclonal antibody against rabbit TF (AP-1) resulted in complete inhibition of intravascular thrombus formation (Pawashe *et al.*, 1994). In a subsequent study the same antibody also improved the performance of the thrombolytic agent tissue plasminogen activator (TPA). Administration of the antibody shortened the TPA clot lysis time and lowered the reocclusion rate when TPA infusion was stopped compared with control rabbits (Ragni *et al.*, 1996). In a third study, Speidel *et al.*, 1995 observed inhibition of coagulation on the intimal surface of the balloon injured rabbit aorta by an anti-TF antibody. These studies demonstrate the importance of TF inhibition as an antithrombotic therapy. However, infusion of monoclonal antibodies in a human clinical setting is a far from ideal solution. The infusion of murine protein may cause immunological problems and, in addition, monoclonal antibodies are unable to discriminate between the extravascular constitutively expressed TF and the induced TF expressed in monocytes. This highlights the need for a neater more specific therapy.

1.6.2 Tissue Factor Pathway Inhibitor (TFPI)

Tissue factor pathway inhibitor is an endogenous multivalent kunitz-type proteinase inhibitor expressed primarily by the microvascular endothelium. It is thought to be the major physiologic inhibitor of TF-induced coagulation, producing factor Xa-mediated feedback inhibition of the factor VIIa/TF catalytic complex (Broze *et al.*, 1988).

Animal studies have shown that the immunodepletion of endogenous TFPI sensitizes rabbits to DIC induced by TF or endotoxin infusion (Sandset *et al.*, 1991a, 1991b). Conversely, infusion of high, therapeutic concentrations of TFPI in rabbits inhibits the intravascular coagulation induced by TF in rabbits and prevents mortality in a baboon model of *E. coli* sepsis (Day *et al.*, 1990; Creasey *et al.*, 1993). As discussed previously, induced TF expression underlies the pathology of many coagulation disorders. However, it has been shown that in the cell types thought to demonstrate abnormal TF expression TFPI expression is either absent (Werlin *et al.*, 1993) or delayed (Bajaj *et al.*, 1996; Bajaj *et al.*, 1993). Furthermore, although plasma TFPI levels in patients with these diseases are generally normal or high, a number of subjects have been found to have low plasma levels (Girard, 1997). Despite the presence of 2-fold elevated plasma TFPI levels in several clinical conditions with TF induced coagulopathy, several observations have indicated the potential for therapeutic administration of exogenous TFPI to inhibit the TF/VIIa complex. Firstly, relatively high concentrations of TFPI are required to inhibit the TF/VIIa complex without the need to initially generate factor Xa (Callander *et al.*, 1992b) allowing the inhibition of TF/VIIa induced coagulation before it has been initiated. Secondly, the plasma TFPI levels achieved during a severe coagulopathy are in a much lower range than might be needed to have a totally therapeutic effect (Girard 1997). In addition, the TFPI released due to endothelial damage may be C-terminally truncated and, therefore, a less powerful anticoagulant (Broze *et al.*, 1995; Broze *et al.*, 1994; Nordfang *et al.*, 1991). Two forms of recombinant TFPI are currently available for therapeutic purposes: full length rTFPI and the two domain rTFPI in which the third Kunitz domain and the C-terminal region have been deleted. These two forms have different pharmacokinetic and activity profiles making each one suitable for different conditions. Full length rTFPI binds to factor Xa at a much faster rate than the two domain rTFPI resulting in faster inhibition of the TF/VIIa complex (Broze *et al.*, 1995; Nordfang *et al.*, 1991) making it more useful when rapid inhibition of TF induced coagulation is required. In addition, full length rTFPI is cleared more rapidly from the circulation and so is only useful if targeted to a focal area of injury by an indwelling catheter. In contrast, the two domain rTFPI persists in the circulation for a

longer period (Holst *et al.*, 1996) making it potentially more useful when a more generalized anticoagulant effect is required such as in sepsis. It has been shown by Bregengard *et al.*, 1993 that infusion of two domain rTFPI inhibited endotoxin induced DIC in rabbits. In a baboon model of *E. coli* sepsis administration of full length rTFPI decreased Il-6 levels and decreased mortality (Creasey *et al.*, 1993; Carr *et al.*, 1995). Full-length rTFPI is currently being used in clinical trials in patients with sepsis and DIC and in those following microvascular surgery. It could also be used in other diseases complicated by TF-induced coagulation such as unstable angina and to prevent thrombosis following fibrinolysis.

1.6.3 Active Site Inhibited Factor VIIa

Active site inhibited factor VIIa (VIIai) competes efficiently with factor VIIa for binding to TF (Sørensen *et al.*, 1997), thereby inhibiting the initiation of coagulation. Therefore, factor VIIai is a potential inhibitor of TF/VIIa initiated thrombosis. The anti-thrombotic effect of factor VIIai has been demonstrated in animal models. In baboons recovering from femoral artery balloon angioplasty factor VIIai abolished thrombus formation at sites of vascular injury (Harker *et al.*, 1995; Harker *et al.*, 1996). In addition, in an atherscleroticrabbit arterial injury model, factor VIIai was shown to reduce angiographic restenosis and neointimal hyperplasia (Jang *et al.*, 1995) Furthermore, topical administration of factor VIIai was seen to increase arterial patency rated in a rabbit model of arterial thrombosis (Arnlijots *et al.*, 1997)

1.6.4 Molecular Inhibition of TF

The main problem with the strategy of generalised inhibition of the mature TF protein, may be the lack of complete understanding of the *in vivo* distribution of TF under normal and pathological conditions. In addition, its physiological roles beyond cofactor function in coagulation are poorly understood. As previously mentioned, the TF structure bears strong similarity to the cytokine family of receptors and therefore, it is possible that TF may be involved in immunologic responses of unknown nature

(Edgington *et al.*, 1992). In addition, the brain contains very large amounts of TF, and it is possible that uncontrolled, generalised inhbition of TF activity may result in excess bleeding. Insights into the molecular mechanisms that regulate TF expression are of interest in the pursuit of novel therapies to attenuate aberrant generation of thrombin. Specific inhibition of induced TF in monocytes at the genetic level, may provide a more specific means of blocking intravascular TF production.

1.6.4.1 Transcriptional Inhibition

Elucidation of the molecular mechanisms that regulate TF gene expression, particularly in monocytes and macrophages, may permit the development of therapeutic strategies that will inhibit inducible TF expression associated with various thrombotic diseases. The expression of TF on the surface of monocytes and endothelial cells requires transcriptional activation of the TF gene and translation of TF specific mRNA transcripts into protein that is translocated to the plasma membrane (Edgington *et al.*, 1991). Intervention at the level of transcription provides a more precise method of specifically inhibiting the TF gene. Strategies aimed at blocking activation of the transcription factor NF-κB, involved in control of TF expression, are being explored in the context of cardiopulmonary bypass surgery (Boyle *et al.*, 1996). In addition, LPS induction of TF can be inhibited in monocytes and macrophages by immunosupressive agents such as Il-4 (Ramani *et al.*, 1993a) and Il-10 (Ramani *et al.*, 1993b), and also by agents which increase the level of cAMP such as dibutyryl cAMP (Prydz *et al.*, 1980; Ollivier *et al.*, 1993; Mackman *et al.*, 1994; Galdal *et al.*, 1984). It is thought that the increase in cAMP may reduce TF mRNA levels by reducing the rate of gene transcription (Ollivier *et al.*, 1993; Ollivier *et al.*, 1996)

1.6.4.2 Antisense Oligonucleotides (ASODNs)

Perhaps the most specific method of inhibiting the induced expression of TF in monocytes/macrophages is at the level of translation. Antisense oligonucleotides

(ASODN) are short, traditionally 15-25 bases long, single stranded DNA fragments, which are designed to specifically hybridize to a target mRNA and temporarily prevent its translation into protein. Sequence specific interference with mRNA function by complementary oligonucleotides was first proposed over two decades ago by Belikova *et al.*, 1973. The first example of specific inhibition of gene expression by an ODN was reported by Zamecnik and Stephenson in 1978, who demonstrated that a short ODN inhibited Rous-sarcoma-virus replication in cells culture. Since then, many groups have investigated the potential for inhibition of a range of specific proteins by antisense oligonucleotides. The majority of these studies have focused on viral or cancer targets for ASODN therapy. To date, only one group has investigated the possibility of inhibiting TF induction using antisense oligonucleotides. Stephens and Rivers, 1998 have shown that co-addition of an ASODN to the TF mRNA and LPS to isolated human monocytes results in a 80% inhibition in TF activity when compared to control ASODNs. This study, although promising, did not attempt to directly measure a reduction in TF protein levels. The authors also found it necessary to conjugate their ASODN to a CD14 antibody to specifically target monocytes. During the course of this thesis, we have further investigated the potential of naked, unconjugated ASODN targeted to the TF mRNA, at inhibiting induction of TF protein in monocytes in a whole blood model.

1.6.4.3 Transgenic Knockouts of TF

In contrast to the other proteins involved in coagulation, no congenital deficiency of TF has been reported, suggesting that it is essential in development. The development of Transgenic Knockout (TKO) technology has made it possible to specifically inactivate a chosen gene and examine the consequences of its absence in a murine model. Complete inactivation of the TF gene was lethal, virtually all TF deficient embryos dying in utero between 9.5 and 10.5 days post coitum (Bugge *et al.*, 1996; Carmeliet *et al.*, 1996). In a separate study, approximately 15%of embryos survived past day 10, but none completed gestation (Toomey *et al.*, 1996). Inactivation of the TF gene results in abnormal circulation from the yolk-sac to the embryo beyond day

8.5 (Carmeliet *et al.*, 1996). The fatal effects of TF gene disruption along with the fact that TF is expressed early in organogenesis before the appearance of detectable amounts of factor VIIa (Luther *et al.*, 1996) suggest that TF has other, as yet unknown roles aside from initiator of coagulation.

1.7 Aims of the Thesis

The importance of TF at initiating the coagulation cascade and the role of induced TF expression on monocytes in producing aberrant clot formation in a variety of pathophysiological processes has so far been discussed. In addition, the therapeutic benefit of carefully controlled TF inhibition has been described. There is an obvious need for more specific and localized therapeutic inhibition of TF at the cellular level. Therefore, the experiments discussed in this thesis aim to characterize the LPS induced expression of TF on monocytes in whole blood. Initial experiments used *E. coli* LPS but in chapter 7, TF induction by a variety of bacterial LPSs including *Bacterioides fragilis* LPS (the most prevalent bacteria in the gut) was investigated. Ultimately, the work outlined in this thesis, investigates the potential of the monocyte as a target for antisense inhibition of LPS-indcued TF, with the view to producing a novel and specific antithrombotic therapy.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Flow Cytometry

2.1.1 Principle of Flow Cytometry

The flow cytometer, analyses cells as they travel in a pressurised moving stream past a fixed laser beam. As a cell passes in front of the laser, several measurements are made based on the physical characteristics of the cell. Using an Optical-to-Electronic system, the flow cytometer records how a cell interacts with a focused laser beam in terms of the cell's ability to scatter the incident light and emit fluorescence. This information is collected and transmitted to the computer. The size and granularity of the cell can be determined by virtue of its light scattering properties. The larger the cell, the greater its forward light scattering abilities (FSC) and the more granular or complex the cell, the greater its side light scattering ability (SSC) (Fig 2.1). The light scattering ability of the cells is displayed as a characteristic 'dot-plot' pattern (Fig 2.2).

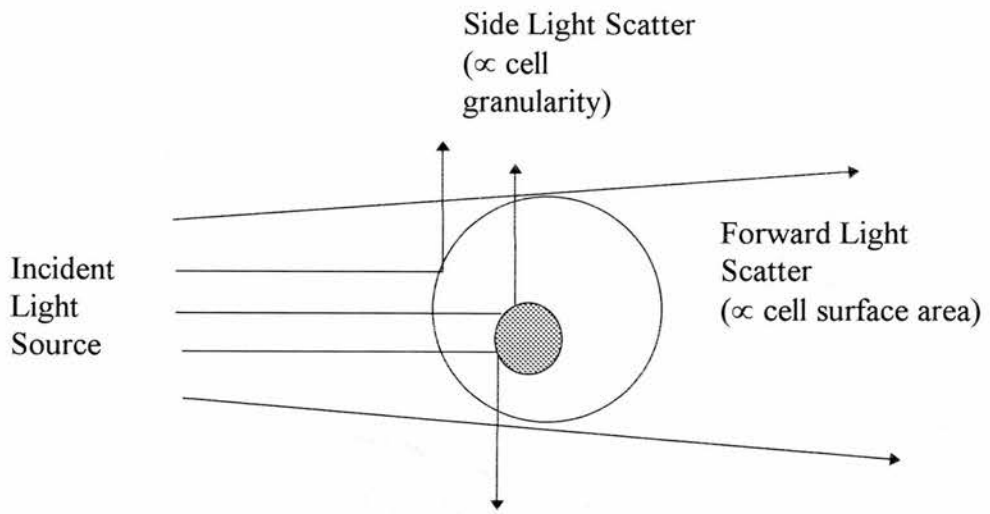


FIGURE 2.1 Light Scattering Properties of Cells
The size and granularity of cell can be determined by its light scattering properties and detected by flow cytometry. Forward light scatter is proportional to cell surface area whilst right angle scatter is proportional to granularity.

In addition to the size and shape of cells, the flow cytometer can detect fluorescent molecules within the cell or on the cell surface. In staining, the cell will bind a fluorescent dye and/or a fluorochrome conjugated antibody in an amount proportional to the quantity of the binding constituent (eg cell surface antigen). Alternatively the cell may internalise quantities of fluorescently labelled molecules . The cell's emitted fluorescence intensity will then be proportional to the fluorescing cellular constituent. The fluorochrome absorbs energy from the incident laser light source and subsequently releases it by emitting photons of a longer wavelength called fluorescence (Fig 2.3). This fluorescence is detected on one of three channels (FL1, FL2 or FL3) depending on the wavelength.

2.1.2 Cell-Type Detection

Peripheral blood leukocytes (PBLs) display a characteristic dot-plot pattern when analysed by flow cytometry. Lymphocytes are small, agranular cells with an uncomplex interior and thus appear on the dot plot with a low forward and side scatter (Fig 2.3). Neutrophils are large complex cells with large granular, lobed nuclei and, as such, appear on the dot plot with a high forward and side scatter pattern (Fig 2.3). Monocytes are also large cells but with smaller more regular lobed nuclei. Therefore, they have an easily distinguishable position on the dot-plot appearing with a high forward scatter, and a side scatter somewhere between that of a lymphocyte and a neutrophil (Fig 2.3). Consequently, a particular cell type can be singled out by virtue of its position on the dot-plot screen and 'gated' so that its particular fluorescence characteristics can be examined. During the course of experimentation, certain treatments may cause certain cell types, in particular monocytes, to change shape and move outwith their gate. In these situations it is necessary to adjust the gate settings to 'keep track' of the monocytes.

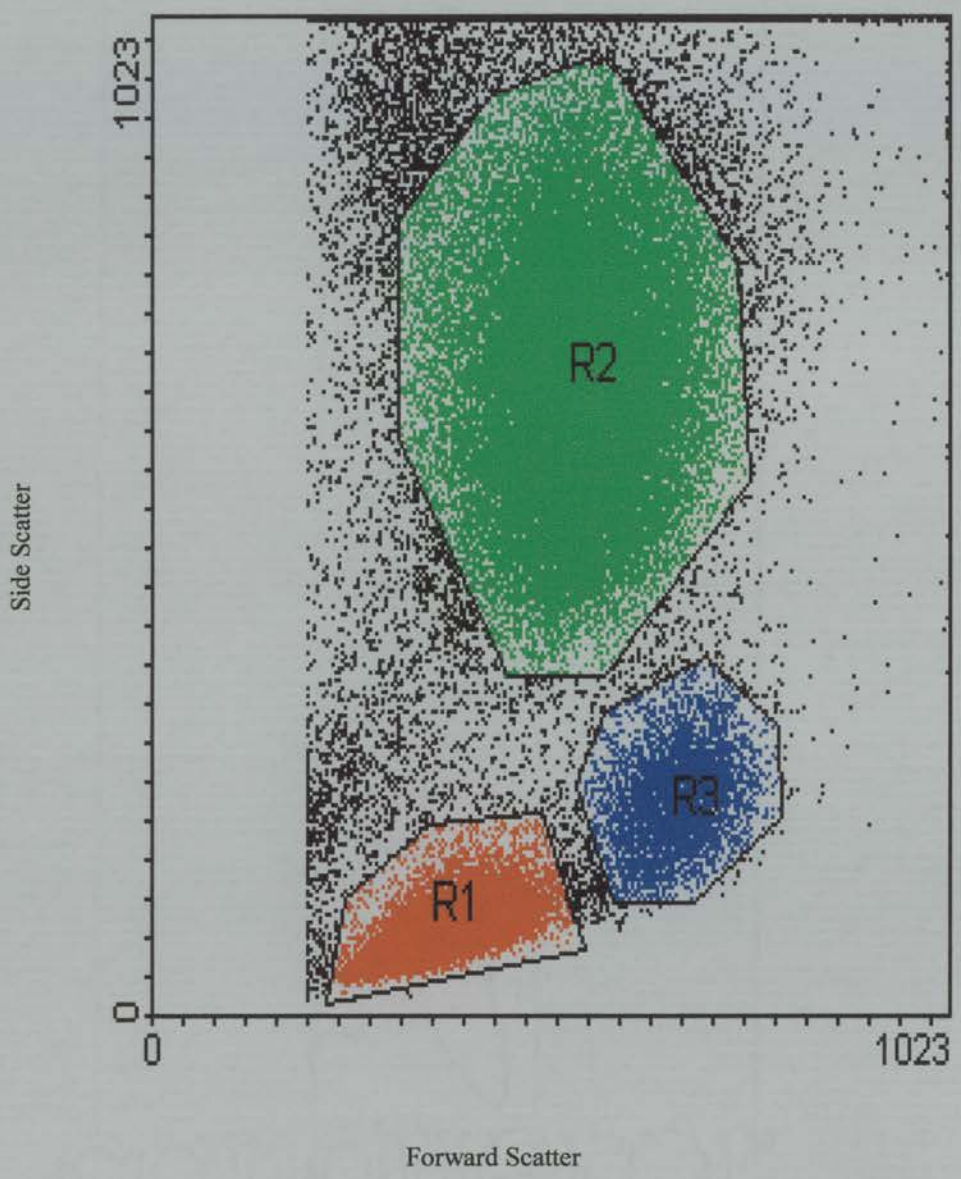


FIGURE 2.2 Characteristic Dot Plot of Peripheral Blood Leukocytes.

R1 Lymphocytes
R2 Neutrophils
R3 Monocytes

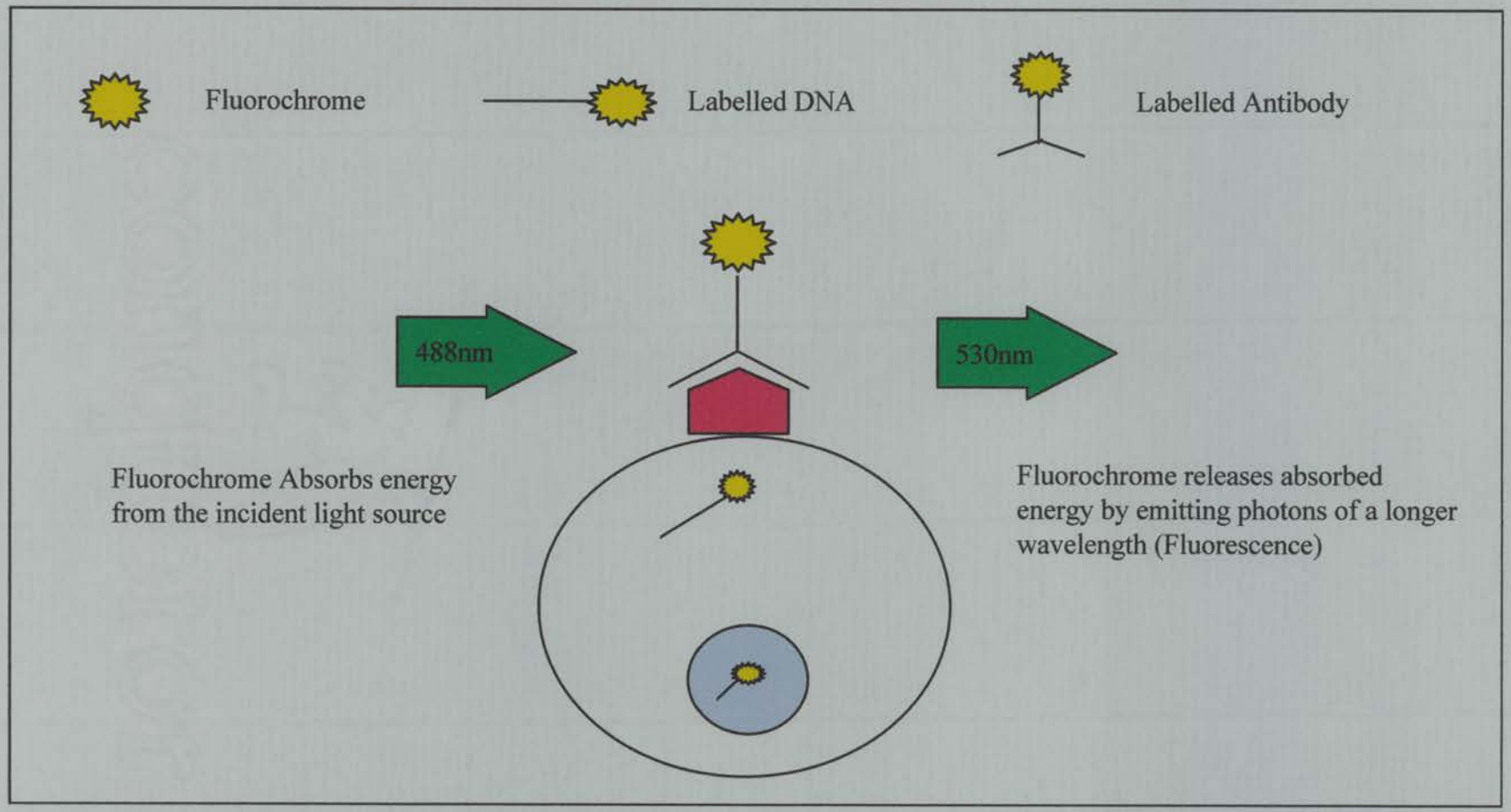


FIGURE 2.3 Fluorescence Detection. Fluorescently labelled antibody on the cell surface or fluorescent molecules taken up by cells absorb energy from the incident laser light and emit fluoresence which is detected by the flow cytometer. The wavelengths of light absorbed and emitted by FITC are shown.

2.1.3 Cell Analysis

Having identified and gated the cell population of interest, the fluorescence profile of these cells is displayed as a fluorescence histogram, whereby the cell count is plotted against the fluorescence intensity of the cells. The relative fluorescence characteristics of the cells can then be quantified in one of two ways:

1. **Geometric Mean Fluorescence**. As the fluorescence intensity is often plotted on a log scale, the 'Geometric Mean Fluorescence' (GMF) is a measure of the overall mean fluorescence of the cells selected taking into account the logarithmic scale. (Fig. 2.4)

2. **Percentage Positive Cells**. This analysis method has been used by Leatham, *et al.*, 1995 and Amirkosravi *et al.*, 1996. Using an isotype control antibody (an antibody which does not bind to the protein of interest but is of the same isotype as the test antibody) or an unlabelled analogue of the fluorescent molecule to be tested, a fluorescence histogram is created and a marker set so that this marker contains a known percentage of cells. In the experiments to follow, this positive delineator is set so that 3% of isotype control labelled cells are counted as positive and this is designated 'background'. Thus, when the true molecule or antibody is used, the percentage of cells falling inside the marker (percent positive cells) can be determined. (Fig 2.4).

The percentage positive cells method was used to measure TF antigen on monocytes as recommended by Amirkosravi et al., 1997. This method enables a permanent 'background' delineator to be set (in this case 3% positive cells). This delineator can then be used as a template for all subsequent experiments. Cells in the monocyte gate were periodically checked for CD14 positivity and were found to be >95% CD14 positive each time they were tested. A dual staining method for simultaneous measurement of GEM-91 fluorescence and CD14 antigen was not practical in this

case as the FAM-label fluorescence was of an intensity that consistently quenched the CD14-PE label.

All flow cytometric experiments were carried out on a Fluorescence Activated Cell Scanner (FACScan) benchtop flow cytometer using FACSFlow™ optimized sheath fluid (Becton Dickinson Immunocytometry Systems, San Jose, California, USA) Analysis was carried out using the Macintosh driven Cell Quest™ sofware specially designed for use with Becton Dickinson flow cytometers.

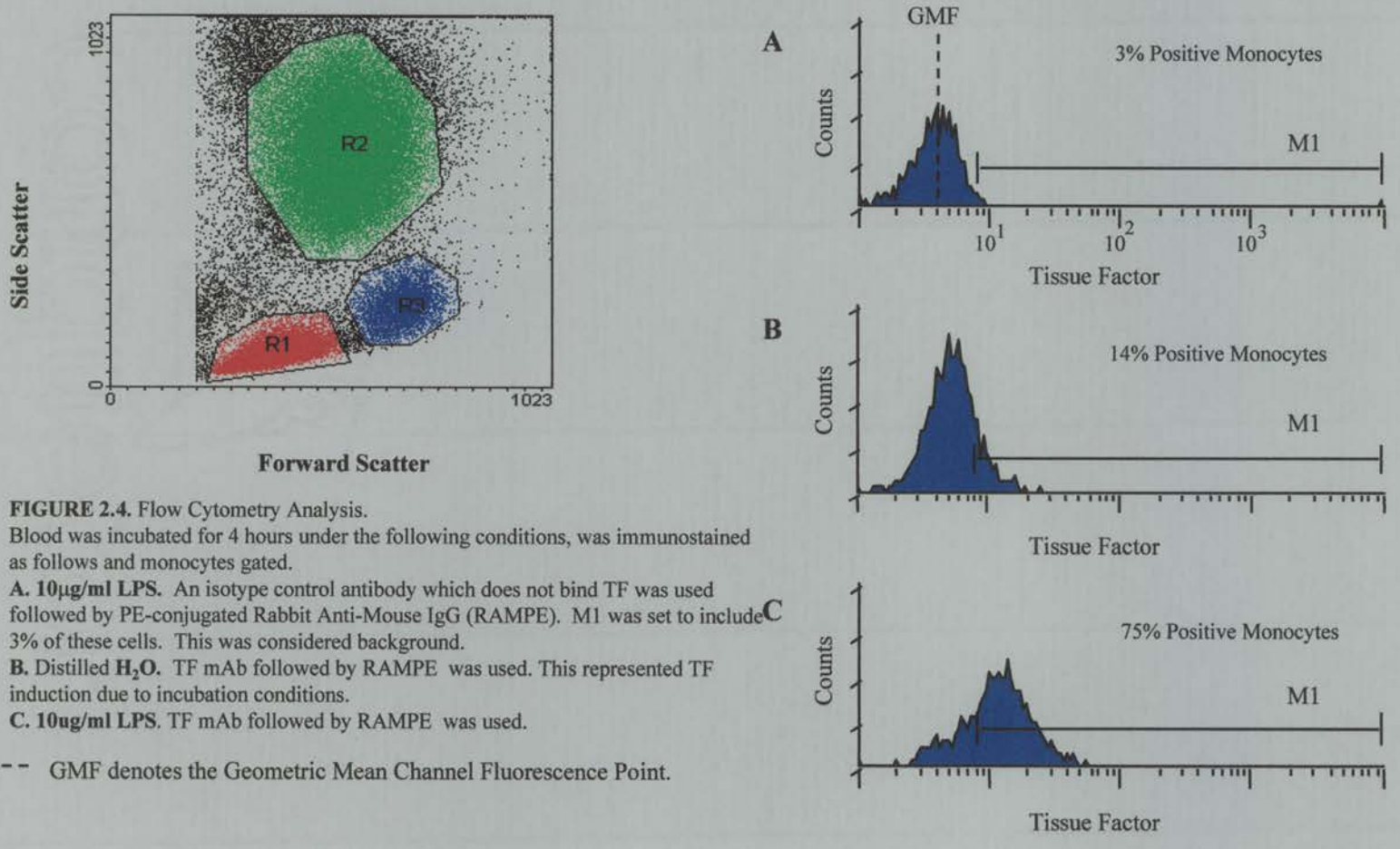


FIGURE 2.4. Flow Cytometry Analysis.
Blood was incubated for 4 hours under the following conditions, was immunostained as follows and monocytes gated.

A. 10μg/ml LPS. An isotype control antibody which does not bind TF was used followed by PE-conjugated Rabbit Anti-Mouse IgG (RAMPE). M1 was set to include 3% of these cells. This was considered background.

B. Distilled **H_2O.** TF mAb followed by RAMPE was used. This represented TF induction due to incubation conditions.

C. 10ug/ml LPS. TF mAb followed by RAMPE was used.

--- GMF denotes the Geometric Mean Channel Fluorescence Point.

2.1.4 Fluorescence Conjugates Used

Some antibodies and ODNs used were conjugated to a fluorochrome for detection by flow cytometry. The fluorochromes used are listed below. Suppliers of the conjugates are listed in the text.

2.1.4.1 FITC (Fluorescein IsoThioCyanate)

This fluorochrome absorbs light at the 488nm wavelength and emits in the green 530nm wavelength. It is, therefore, detected using the FL-1 channel on the flow cytometer.

2.1.4.2 FAM (Fluorescein Addition Monomer)

Like FITC, FAM aborbs light at the 488nm wavelength and emits in emits in the green 530 nm range and so can be detected in the FL-1 channel on the flow cytometer.

2.1.4.3 R-Phycoerythrin (R-PE)

Like FITC and FAM, R-PE excites at 488nm but it emits fluorescence in the orange range (575nm). Therefore, it is detected using the FL-2 channel.

2.1.4.4 Quantum Red (PE-Cy5)

Quantum red is a tandem fluorochrome in which a small organic dye, Cy5 is covalently linked to R-Phycoerythrin. The PE absorbs light energy at 488 nm and emits in the excitation range of Cy5 which acts as the acceptor dye. The complex then emits at 670 nm. This can be detected in the red FL-3 channel.

2.1.5 Isolation of Peripheral Blood Leukocytes

Blood samples were collected from healthy, consenting volunteers (staff at the Department of Haematology, Royal Infirmary of Edinburgh) by clean venepuncture into heparin-coated tubes (300ul heparin per 3ml blood) or citrated vacutainers. The red blood cells were lysed by the addition of 4-10 volumes of red cell lysis buffer (150mM ammonium chloride, 10mM potassium bicarbonate, 0.1mM EDTA) for 3-5 minutes at room temperature. Cells were then centrifuged at 1000g for 5 minutes at 4°C and the cell pellet resuspended in 5 volumes of red cell lysis solution. After a final centrifugation step, (400g for 5 minutes), cells were finally resuspended in culture medium (QBSF51, Sigma Chemical Company, Poole, UK), counted in the presence of trypan blue, and plated out at a final density of $1x10^6$ cells per ml of culture medium. All incubations were carried out in a humidified atmosphere of 95% air, 5% CO_2.

2.1.6 Immunostaining

The concentrations of all antibodies used were suggested by Prof. T. Edgington, Scripps Research Institute, La Jolla, and were checked by titration experiments in both isolated monocytes and monocytes in whole blood. The primary TF mab was used at a concentration of 10μg/ml in a final volume of 100μl. 100μl of a 1/20 dilution in PBS of phycoerythrin conjugated rabbit anti-mouse IgG fab(2') fragment (RAMPE) (Dako, Ltd) was used as the detection antibody.

2.1.6.1 Direct Immunostaining (Isolated Cells)

A 100μl aliquot of cells ($1x10^7$) was placed in a sterile polypropylene tube and to it was added the appropriate monoclonal antibody preconjugated to its fluorochrome. This was incubated for 30 minutes at 4°C in the dark. The cells were then washed twice in washing solution; 10ml fetal calf serum, 25ml 4% sodium azide in 465ml Phosphate Buffered Saline(PBS), (made by dissolving 2 tablets in 400ml distilled

water), and resuspended in 300µl washing solution before being immediately analysed by flow cytometry. All reagents were purchased from Sigma Chemical Company unless otherwise stated.

2.1.6.2 Direct Immunostaining (Whole Blood)

100µl of whole blood was placed in polypropylene tubes containing the appropriate preconjugated monoclonal antibody and incubated at 4°C for 30 minutes in the dark. The red blood cells were lysed by the addition of 4ml of red cell lysis solution as above, washed twice in washing solution (as above), and fixed overnight by the addition of 300µl 1% paraformaldehyde in PBS and 300µl washing solution. The cells taken from whole blood incubations post red-cell lysis were fixed in paraformaldehye overnight as this removed any red blood cell contamination and improved the separation of the cell populations on the FACS plot. In contrast, isolated cultured cells were not fixed as they were less robust than cells from whole blood incubations and changed morphology so as to be unrecognisable on the FACS plot. There was no difference in the fluorescence characteristics between fixed and unfixed cells (Fig 2.5).

2.1.6.3 Indirect Immunostaining (Isolated Cells)

A 100µl aliquot of cells ($1x10^7$/ml) was placed in a sterile polypropylene tube and to it was added the appropriate primary monoclonal antibody. This was incubated for 30 minutes in the dark at 4°C and was then washed twice in washing solution as above. The cells were then resuspended in 100µl of 1/20 dilution in PBS of phycoerythrin conjugated rabbit anti-mouse IgG fab(2') fragment (RAMPE) (Dako, Ltd) detection antibody. The fab(2') fragment was used to minimalise binding to fc receptors on the monocytes and neutrophils in the cell suspension. This was incubated for 30 minutes at 4°C in the dark. The cells were finally washed twice in washing solution and resuspended in 300µl of washing solution before being analysed by flow cytometry.

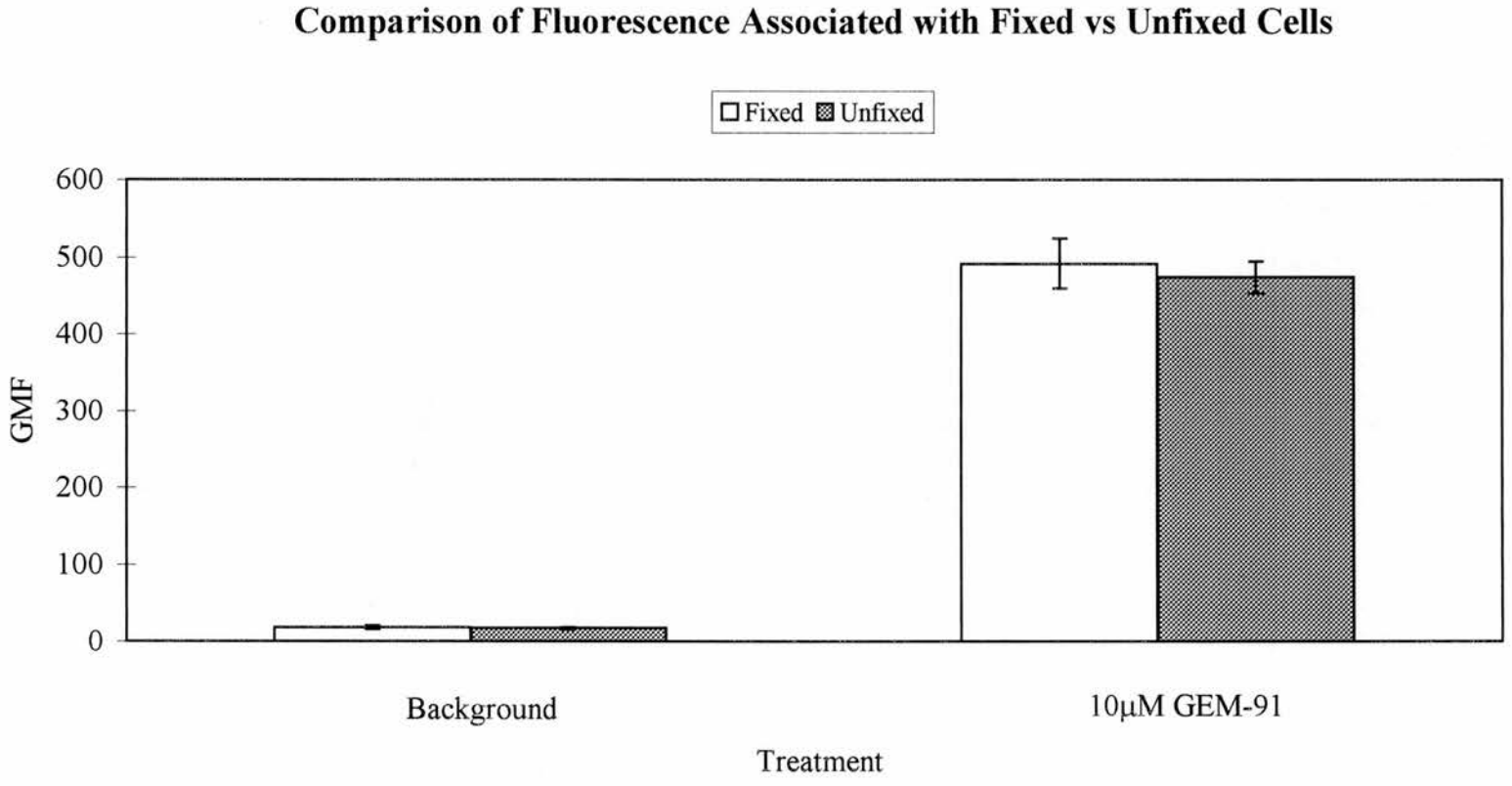


Figure 2.5 Comparison of Fluorescence Associated with Fixed vs Unfixed Cells. Untreated (background) cells and GEM-91 treated cells were either fixed in 1% paraformaldehyde solution overnight or analysed immedictely by flow cytometry . Bars represent the mean of 5 separate experiments +/- standard error of the mean. It can be seen that overnight fixation of the cells had no effect on their fluoresence characteristics.

2.1.6.4 Indirect Immunostaining (Whole Blood)

100µl of whole blood was placed in polypropylene tubes containing the appropriate primary monoclonal antibody. This was incubated a 4°C for 30 minutes in the dark. The whole blood was then lysed by the addition of 4mls red blood cell lysis solution (as above) for 3-5 minutes at room temperature before being centrifuged for 10 minutes at 400g at 4°C. The cell pellet was then washed twice in washing solution at 4°C before being incubated with the detection antibody RAMPE as above. The cells were then washed twice in washing solution and fixed with 300µl 1% paraformaldehyde solution in PBS and 300µl washing solution overnight.

2.1.7 Statistical Analysis

The raw data from all paired experiments were analysed by a repeated measures analysis of variance (ANOVA) followed by an appropriate post test. If each test condition was being compared with one control a Dunnett's post test was used. If test groups were being compared with each other a Bonferroni post test for multiple comparisons was used. If experiments were not paired as when sample size differed, raw data was not used and group means were compared by a one way ANOVA followed by an appropriate post test. All statistical analyses were performed using the Graph-Pad Instat programme, and statistical advice was given by Ms Kay Penny, University of Edinburgh, Medical Statistics Department.

CHAPTER 3

UPTAKE OF ANTISENSE OLIGODEOXYNUCLEOTIDES IN PERIPHERAL BLOOD LEUKOCYTES

3.1 Introduction

3.1.1 The Antisense Strategy

The concept of the antisense (AS) strategy is a straightforward one: inhibition of a particular target protein using single stranded oligodeoxynucleotides (ODNs) of a sequence complementary to that of the specific messenger RNA (mRNA) (Fig 3.1) This strategy is so called due to its reliance on the formation of reverse complementary (antisense) Watson-Crick base pairing between the antisense construct and the mRNA to be inhibited (Fig 3.1). It is the specificity of this base pairing that allows the potentially selective targeting of a particular mRNA. Helene and Toulme declare that the sequence specificity of the antisense technique is based on the fact that a sequence of about 13 bases in RNA is unique in the human genome (Helene & Tolume, 1990). However, although the probability of a 13 base sequence reoccurring in the genome as a whole is low, in view of the non-random nature of the human genome, sequences of bases are more likely to reoccur in DNA coding for proteins in the same class, such as in receptor families. There is, therefore, the possibility of an antisense molecule having more than one target. The idea that gene expression may be modulated by the use of exogenous nucleic acid derivatives was first alluded to by Paterson et al in 1977 when they used single stranded DNA to inhibit translation of a complementary RNA in a cell-free system. The first indication that the antisense mechanism might be used therapeutically came in 1978 when Zamecnik and Stephenson demonstrated that a 13 nucleotide DNA molecule complementary to the Rous sarcoma virus could inhibit viral replication in culture. In the 1980s, Simons and Kleckner, 1983 and Mizuno *et al.*, 1984, demonstrated the existence of naturally occurring antisense RNAs in prokaryotes and showed that these molecules were involved in regulating expression of their corresponding genes. Furthermore, in eukaryotic cells, naturally occurring antisense transcripts also exist which are involved in physiological gene regulation (Farrell and Lukens, 1995; Kimelman and Kirschner, 1989). These observations suggested that the use of reverse complementary antisense molecules might be of use therapeutically. Recently, synthetic antisense molecules

have been artificially introduced into cells to inhibit gene function (Zhang *et al.*, 1996), and it has been shown that antisense ODNs microinjected into the cytoplasm of cells inhibit their target protein in a specific and efficient manner (Wagner *et al.*, 1995).

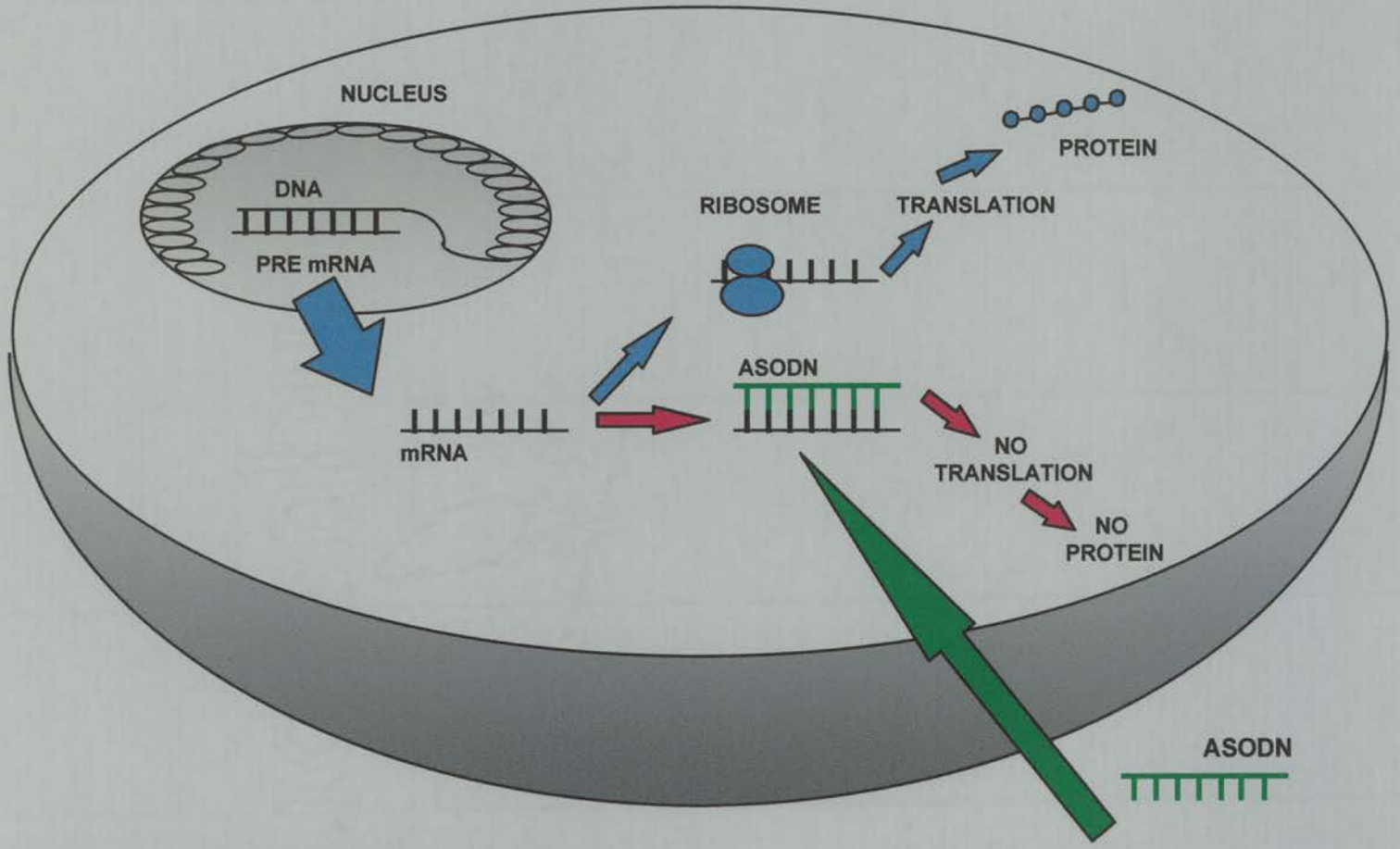
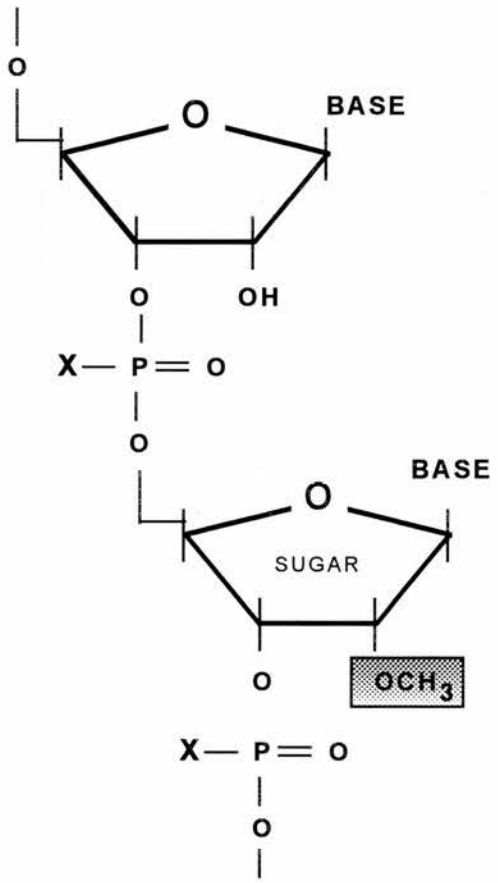


FIGURE 3.1 Overview of the Antisense Mechanism of Protein Inhibition.
ASODNs enter cells and bind, by Watson and Crick base pairing, to a specific target mRNA. This arrests translation of the target mRNA and prevents synthesis of the target protein. (For more detailed discussion of the mechanisms involved see chapter 6).
Adapted from website http://www.hybridon.com.

3.1.2 Antisense ODNs

Antisense oligodeoxynucleotides (ASODNs) are short, typically 10-30 base, single stranded lengths of DNA which are designed to hybrize to a specific mRNA and prevent translation of a single target protein (Fig 3.1). Antisense oligodeoxynucleotides need to meet certain physical requirements to make them useful. Firstly, they need to cross cell membranes and secondly they must be able to hybridise with their intended target. The ability of an ODN to form a stable hybrid is a function of its binding affinity and sequence specificity. Binding affinity is a function of the number of hydrogen bonds formed between the ODN and its target mRNA. Thus, as the G-C base pairing utilises 3 hydrogen bonds as opposed to the two between C and T, it may be concluded that hybridisation stability will increase with G-C content of the ASODN. At physiologic conditions, it has been estimated that at least 12 bp need to form in order to form a stable hybrid with a phosphodiester backbone (Khan *et al.*, 1993). While increased length increases oligo affinity for its target, it is thought that the non-specific effects of ASODNs, such as protein aptamer binding or hybridization to random base sequences increase with increasing G-C content and ODN length. Unmodified phosphodiester ODNs were the first generation molecules to be investigated and have been investigated for use *in vitro* and *in vivo*. However, they are extremely sensitive to attack by endogenous nucleases, the half life of phosphodiesters in growth medium containing 10% calf serum being about 30 minutes (Crooke *et al.*, 1992). Next generation antisense molecules were subsequently designed so that the internucleotide link was more resistant to attack. This was accomplished by replacing one of the non-bridging oxygen atoms in the phosphate group with either a sulphur or a methyl group (Fig. 3.2). This type of modification results in a phosphorothioate (Zon., 1995) or a methylphosphonate (Crooke., 1991) respectively (Fig. 3.2). It has been the phosphorothioate molecules that have been most extensively investigated both in the laboratory and in several clinical trials (Gewirtz *et al.*, 1996; de Fabritis *et al.*, 1995; Webb *et al.*, 1997). In addition to being resistant to nuclease attack, the phosphorothioates are negatively charged molecules and so are water soluble. Furthermore, they permit RNase H

activity when in the duplex (see below). However, the polyanionic nature of these molecules can impair cellular uptake because of the negative charge at the cell surface.



Common Linkage Modifications:	
x = OH	Phosphodiester
x = OCH 3	Methyl Phosphonate
x = S	Phosphorothioate

Common Sugar Modifications:
2'-O- methyl group(as shown)
2'-O-methyoxyethoxy-group

FIGURE 3.2. Common Chemical ODN Modifications. Common modifications of the phosphodiester linkage and sugar moieties are shown.
Adapted from Gewirtz *et al.*, 1998

3.1.3 Cellular Uptake of Phosphorothioate ODNs

Finding effective methods to induce efficient uptake of ODNs into cells is an active area of current research, and is crucial to the success of any ODN based pharmacology (Gerwitz *et al.*, 1998). In addition to spontaneous uptake of naked DNA (Nestle *et al.*, 1994), many different strategies have been employed to increase

ASODN uptake. Microinjection of ODNs has been used successfully by many laboratories (Dagle *et al.*, 1991; Kola *et al.*, 1995; Leonetti *et al.*, 1991) but is obviously of little use clinically, except perhaps in *in vitro* fertilization. Other commonly employed strategies may be classified as those which are aimed at modifying the target cell, typically by permeabilizing the cell membrane, and those which seek to directly or indirectly modify the permeation properties of the ODN. Physical disruption of target cell membranes has been carried out by electroporation (Bergan *et al.*, 1996; Flanagan and Wagner, 1997) or by the use of chemical agents which permeablize the cell membrane such as streptolysin (Giles *et al.*, 1995; Bergan *et al.*, 1996; Flanagan and Wagner, 1997). Both of these methods are physically destructive to cells and so are of little use *in vivo*. They are, however useful in *ex vivo* therapies such as bone marrow purging. Calcium has also been used in recent studies to assist uptake of ASODNs into the cell (Wu-Pong *et al.*, 1994). An alternative, less physically extreme strategy for improving delivery of ODNs into cells is to modify the permeation characteristics of the ODN by packaging the DNA in an artificial vector. Such vectors can be cationic lipids which can be manufactured to contain the DNA within a lamelar structure called a liposome, or coat the DNA by charge interaction (Lewis *et al.*, 1996; Juliano *et al.*, 1992; Behr, 1994). However, these lipid carriers often prove to be more cytotoxic than naked DNA. Finally the ODN itself can be modified. One such strategy is to conjugate the ODN either directly or indirectly to a ligand specific for a receptor resident on the target cell type. For example, ODNs have been conjugated to folate, mannose, asialoglycoproteins and tumour-specific antibodies to target haematopoietic, pulmonary alveolar (Liang *et al.*, 1996;) hepatic and a variety of tumour cells (Kuijpers *et al.*, 1993) specifically. Technical problems with this sort of technology have been encountered such as dissociation of the ODN and the carrier (Lu *et al.*, 1994) rapid clearance of antibody conjugated material or biological sequestering in the endosome compartment (Liang *et al.*, 1996; Bonfils *et al.*, 1992) which also limit the effectiveness to this approach.

Despite much research in this field, there is as yet no consensus on the best method of delivery, which may vary between the cell lines or cells used and depend on the

specific modifications made to the ODN. Studies have yielded conflicting results in that different methods, cell lines and ODNs have been used for evaluating internalization of ODN. The one consistent finding has been that all mammalian cells investigated are capable of internalizing phosphorothioate ODNs by an active process. However, the intracellular fate of the ODN is controversial. Some studies suggest that ODNs are internalised by a receptor mediated or adsorptive endocytosis pathway in which the ODN is retained within membrane bound intracellular vesicles (Loke *et al.*, 1989; Stein *et al.*, 1993; Bennett *et al.*, 1993), whilst other studies suggest that the ODN either uses alternative methods for gaining entry into cells such as fluid phase pinocytosis, or escapes from the cytoplasmic vesicles (Wu-Pong *et al.*, 1994; Nestle *et al.*, 1994; Noonberg *et al.*, 1993). More recently it has been proposed that the mechanism of cellular uptake of exogenously administered ODNs occurs by a combination of different mechanisms depending on the concentration of the ODN. It is believed that at ODN doses higher than 1μM, fluid phase endocytosis is responsible whereas at sub micromolar concentrations receptor mediated endocytosis plays a more important role (Beltinger *et al.*, 1995; Benimetskya *et al.*, 1997). So far, the majority of studies have concentrated on uptake of ODNs into cell lines (Iversen *et al.*, 1992; Stein *et al.*, 1993; Temsamani *et al.*, 1994; Tonkinson *et al.*, 1994, Vlassov *et al.*, 1994) which are thought to incorporate ODNs more efficiently than primary cells. Despite its implication for *in vivo* studies, information about spontaneous uptake of naked DNA in primary peripheral blood leukocytes is limited. Currently, only 4 studies examining the uptake of ODN into PBLs have been documented, (Marti *et al.*, 1992, Iversen *et al.*, 1992, Pirruccello *et al.*, 1994, Zhao *et al.*, 1996). Leukocytes are currently important target cells in clinical studies testing antisense ODNs as therapeutics for inflammatory disease including rheumatoid arthritis and Crohn's disease (Bradbury, 1997), for chronic myelogenous leukaemia and myelodysplastic syndrome (Bayever *et al.*, 1993; Nichols, 1995; Skorski *et al.*, 1994) and for human immunodeficiency virus infection (Zhang *et al.*, 1995). It is our hope to utilize ASODNs to modulate the expression of monocyte TF, and so an understanding the uptake mechanisms and intracellular availability of ODNs in peripheral blood leukocytes is important if these molecules are to be used effectively

as a therapy. We have, therefore, investigated the spontaneous uptake kinetics of a naked 25 base pair phosphorothioate antisense molecule, GEM-91, into peripheral blood leukocyte subtypes to assess the feasibility of using a naked, and thus more therapeutically viable, antisense TF molecule as an effective inhibitor of monocyte TF induction.

3.2 Materials and Methods

3.2.1 ODNs

Biotin-, Fluorescein Addition Monomer (FAM) and ^{35}S labelled GEM-91 ($3x10^5$ dpm/μg) were supplied by Hybridon. Polybead carboxylate microspheres (Polysciences Inc. Warrington, PA) were conjugated to **MEG** (a 25-base ODN complementary to GEM-91 synthesized with a 5' primary amine group) which was supplied by Oswel DNA services, Southampton, UK. This conjugation utilised 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Pierce, Chester, UK) in order to generate an amine reactive intermediate on the microspheres (Staros *et al.*, 1986)

3.2.2 Hybridisation of GEM-91 and Calibration of Label Fluorescence

In order to quantify the amount of ODN binding to cells it was first necessary to calibrate the system using ^{35}S labelled GEM-91 (specific activity 940cpm/pg) and MEG-labelled microspheres. MEG is the reverse complementary sequence of GEM. The following protocol was used:

1. Increasing concentrations (0.01-10,000pg) of ^{35}S labelled GEM-91 (940cpm/pg) were added to 100μl aliquots of MEG conjugated microspheres (20,000 spheres/ml) to detect the point of saturation, where all available MEG sites on all spheres were occupied by ^{35}S-labelled GEM-91 (Fig. 3.3). It can be seen from Fig.3.3 that the binding capacity of the spheres was 100,000cpm per 100μl (2000 spheres). Specific activity of ^{35}S-labelled GEM-91 was 940cpm, therefore 100,000/940 = 106pg GEM-91/100μl suspension. There were 2000 spheres/100μl solution so **53fg GEM-91 bound per sphere**.

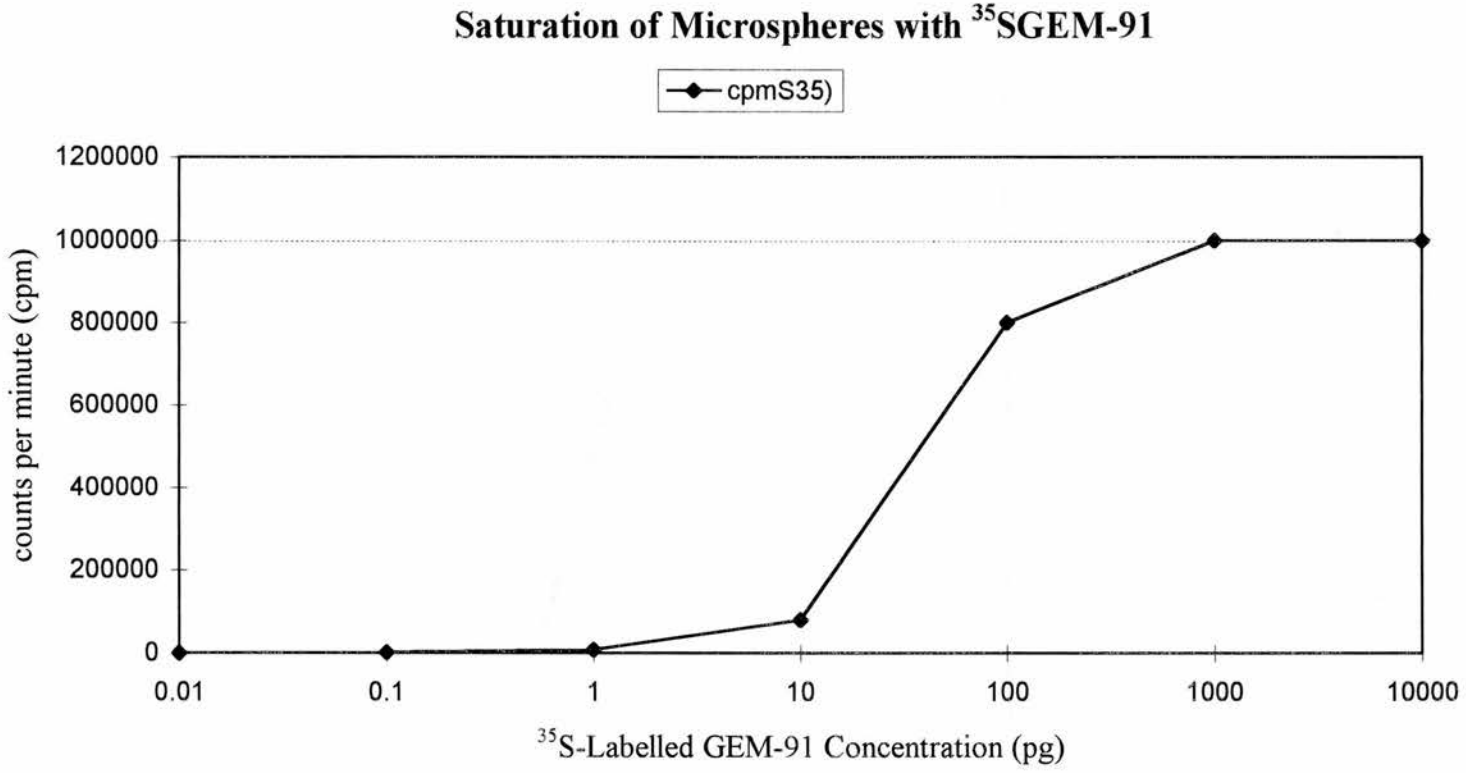


Figure 3.3 Saturation of microspheres with ^{35}S-Labelled GEM-91.
Increasing concentrations of ^{35}S labelled GEM-91 (specific activity 940cpm) were hybridised to 100µl solutions containing 2000 MEG-conjugated microspheres and the activity measured in cpm. Maximum binding capacity was 100,000 cpm per 100µl microsphere suspension, which is equivalent to 53fg GEM-91 bound per microsphere.

2. Spheres were then hybridised with an excess (1μg) of FAM or Biotin-Streptavadin-phycoerythrin- labelled GEM-91 in the same way. The fluorescence intensity (GMF)of the spheres hybridised with FAM or biotin labeled GEM-91 was measured by flow cytometry in triplicate (Table 3.1). FAM labeled GEM-91 was detected on the FL-1 channel and Streptavadin-PE was detected on the FL-2 channel.

Label Used	**GMF of Spheres in Respctive Channels**				
	1	2	3	Mean	SEM
FAM (FL-1)	175	185	171	177	4.2
Biotin/Streptavadin-PE (FL-2)	814	804	827	815	6.7

Table 3.1 Calibration of Label Fluorescence. GMF of microspheres bound with saturating concentrations of FAM-labeled and Biotin/Streptavadin-labeled GEM-91.

Therefore,

GMF (FL-1) 177 ≡ 53fg/sphere. 53/177 = 0.3. GMF units*0.3 = fg/sphere.

GMF (FL-2) 815 ≡ 53fg/sphere. 53/815 = 0.065. GMF units*0.065 = fg/sphere.

It has been assumed in the experiments to follow that human cells bind ODN in a comparable manner to microspheres. Therefore, on the basis of these calibration experiments, it can be concluded that GMF values recorded in the FL1 (FAM) channel must be multiplied by the correction factor 0.3 and in the FL-2 (PE) channel by 0.065 to determine the amount of GEM-91 in fg/cell.

To determine whether hybridisation efficiencies to MEG-conjugated microspheres of all GEM-91 species used were equal, aliquots of each labelled species were denatured at 95°C for 5 minutes before being spun to remove the spheres. The concentration of ODN in the supernatant was then measured by reading its optical density at 260nm. The mean (± standard error mean) concentration of ODN in each supernatant was found to be 30.5±1.4, 30.5±0.8, 29.8±1.1ng per 10^3 spheres for ^{35}S , biotin and FAM

labelled GEM-91 (n=6). It was, therefore, concluded that there were no differences in hybridisation efficiency between the differently labelled ODNs.

3.2.3 Blood Cell Collection and Culture

Blood samples from nine healthy volunteers were collected by clean venepuncture into heparin-coated tubes (300ul heparin per 3ml blood). The mononuclear cells were isolated as described in chapter 2 and were plated out on sterile tissue culture plates (Costar) at a final density of $1x10^6$ cells per ml of culture medium (QBSF51, Sigma Chemical Company, Poole, UK) in a final volume of 5ml. All incubations were carried out in a humidified atmosphere of 95% air, 5% CO_2 at 37°C.

3.2.4 Uptake Studies

3.2.4.1 Preparation of GEM-91 for use in Uptake Studies

Filter sterilised stocks of all GEM-91 solutions were prepared in PBS (Phosphate buffered saline tablets were supplied by Sigma; addition of 1 tablet to 200ml of distilled water produced 0.01M phosphate buffer, 0.0027M potassium chloride and 0.137M sodium chloride, pH 7.4) and stored at -20°C such that the final concentration in the culture could be obtained by the addition of the same volume of drug. Therefore, in all cases, 10µl of GEM-91 was added per 500µl of culture medium.

3.2.4.2 Uptake Methodology and Preparation of Cells for Flow Cytometric Analysis

Uptake of GEM-91 into peripheral blood leukocytes was measured using two methods: Biotin labelled GEM-91 in conjunction with PE-conjugated streptavadin was used to measure extracellular binding of the ODN to the cell surface. Biotin labelled ODN is able to enter the cell whilst the streptavadin is not. Therefore, the

PE-conjugated streptavadin bound only to the cell-surface associated ODN. The original intention of the study was to use FAM labelled GEM-91 to measure total association of ODN with the cell and to subtract the extracellular association as determined by the biotin-streptavadin experiments to derive the intracellular concentrations of ODN. However, during the course of the study it became apparent that the inclusion of the viability stain propidium iodide (PI) in the FAM experiments completely quenched the cell surface associated fluorescence of the FAM label. Sixteen cell samples were chosen at random and incubated with 1μM GEM-91 for 4 hours. The internalised GEM-91 was then measured directly by PI quenching of cell surface bound GEM-91, and was also calculated by subtracting the external GEM-91, as measured by the biotin-streptavadin method, from the total fluorescence. The two different methods were found to give comparable results (Fig.3.4 and Append. 1). This enabled the FAM labelled ODN (with the addition of PI) to be used as a direct measure of intracellular ODN uptake. This phenomenon has also been reported for ethidium bromide quenching of cell surface associated fluorescence (Fattorossi *et al.*, 1989) and has been further confirmed for PI quenching of FAM label by experiments by Cunningham, 1997 (personal communication).

For the subsequent uptake studies, cells from three separate individuals were incubated in the presence of a range of doses of FAM labelled or biotin labelled GEM-91 (0, 0.1, 1.0, 10μM) for up to 4 hours. At set times throughout this culture period (30, 60, 90, 120, and 240 minutes) and at the end of the culture period, cell samples ($5x10^5$ cells) were taken. Cells were centrifuged at 1000g for 5 minutes, washed in 1.5ml PBS, centrifuged and resuspended in fresh PBS. An aliquot of cells treated with FAM-labelled GEM-91 was analysed by flow cytometry followed by an aliquot treated with 40μl 0.25% PI (Sigma Chemical Co) per ml of sample. The geometric mean channel fluorescence value (GMF) was recorded for each sample.

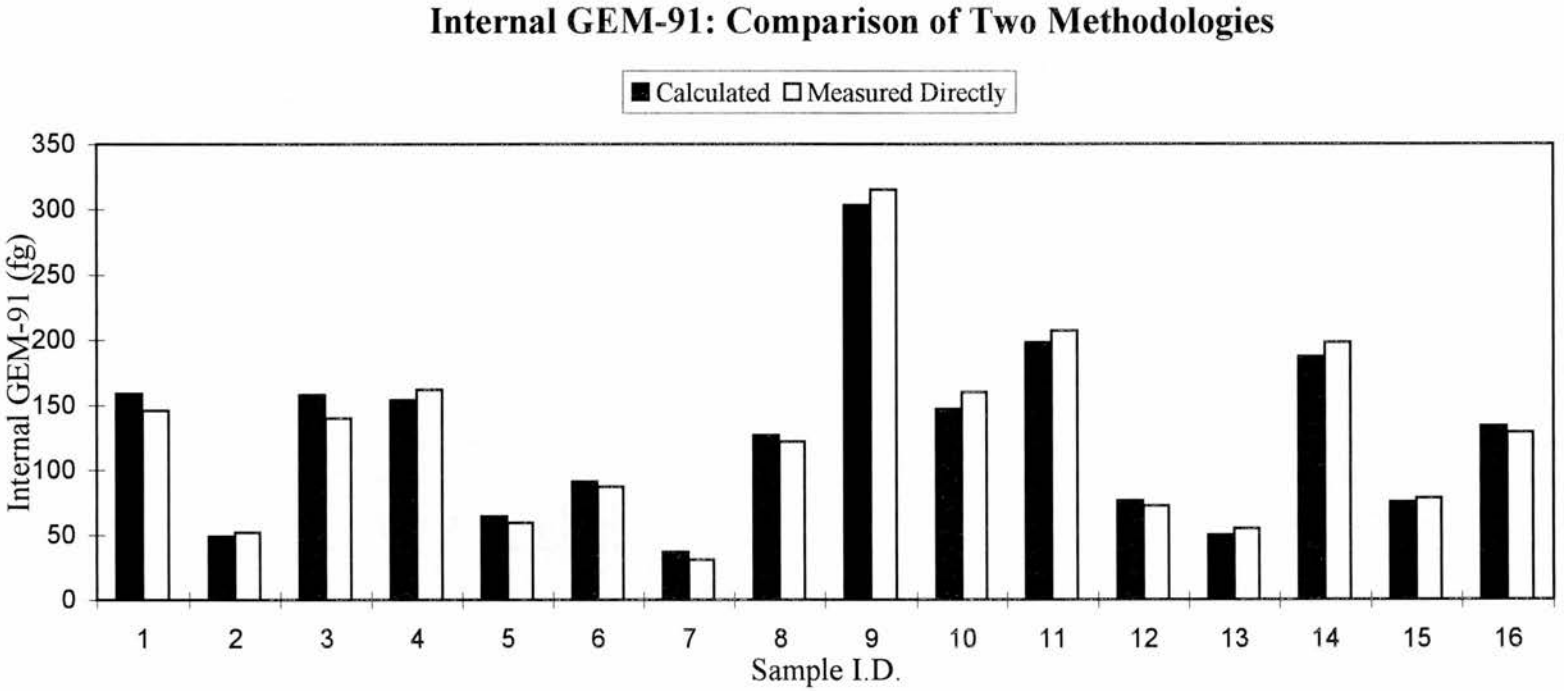


Figure 3.4 Comparison of 2 Methods of Determining Internalised GEM-91.
Intially, it was decided that internalised GEM-91 would be measured indirectly by subtracting the external fluorescence (measured using biotin-labeled GEM-91 detected with PE-conjugated streptavadin) from the total associated fluorescence. However, it became apparent that inclusion of propidium iodide quenched the cell surface fluorescence, leaving the internal fluorescence which could then be measured directly. 16 randomly chosen cell populations were tested and it can be seen that the two methods of determining internalised GEM-91 are comparable.

3.3 Results

3.3.1 Extracellular Uptake of GEM-91 into Cells

3.3.1.1 Total Leukocyte Population

Extracellular uptake in the total leukocyte population was found to be dose-dependent and non-saturating within the range of doses (0.1 to 10μM GEM-91) studied (Fig. 3.3). The highest levels of extracellular association were detected at the earlier timepoints (≤1h), with significant decreases being observed over time. In the presence of 10μM GEM-91, the mean concentration of GEM-91 in unfractionated peripheral blood leukocytes after 30 minutes of culture was 425±112 fg GEM-91/cell with a maximum individual value of 643fg GEM-91/cell. After 4 hours in culture, the mean concentration of GEM-91 had decreased to 115±30 fg GEM-91/cell with a minimum individual value of 80fg GEM91/cell. (Fig 3.3)

3.3.1.2 Lymphocytes

Lymphocytes had lower levels of cell surface associated GEM-91 than the unfractionated PBLs. The mean concentration of GEM-91 was approximately 70fg GEM-91/cell at the highest dose (10μM) after 0.5 h in culture. However, unlike monocytes and neutrophils, lymphocytes showed no measurable decrease in extracellular binding over the timecourse measured (up to 4 hours). (Fig 3.4).

3.3.1.3 Monocytes

Monocytes showed the greatest proportion of total GEM-91 extracellular association. The mean concentration of GEM-91 on the monocyte cell surface was approximately 500fg GEM-91/cell after 0.5 hours of culture in the presence of 10μM GEM-91. This concentration dropped dramatically to approximately 60fg GEM-91/cell after 4 hours, despite the continuing presence of GEM-91 in the cultures (Fig 3.5).

3.3.1.4 Neutrophils

Neutrophils showed a high level of extracellular GEM-91 binding. The mean concentration of GEM-91 on the neutrophil cell surface was approximately 350fg GEM-91/cell after 0.5 hours of culture in the presence of 10μM GEM-91. This concentration decreased to approximately 135fg GEM-91/cell after 4 hours. This decrease was less marked than in monocytes (Fig 3.6).

Raw data for extracellular binding in all cell types can be found in Appendix B1.

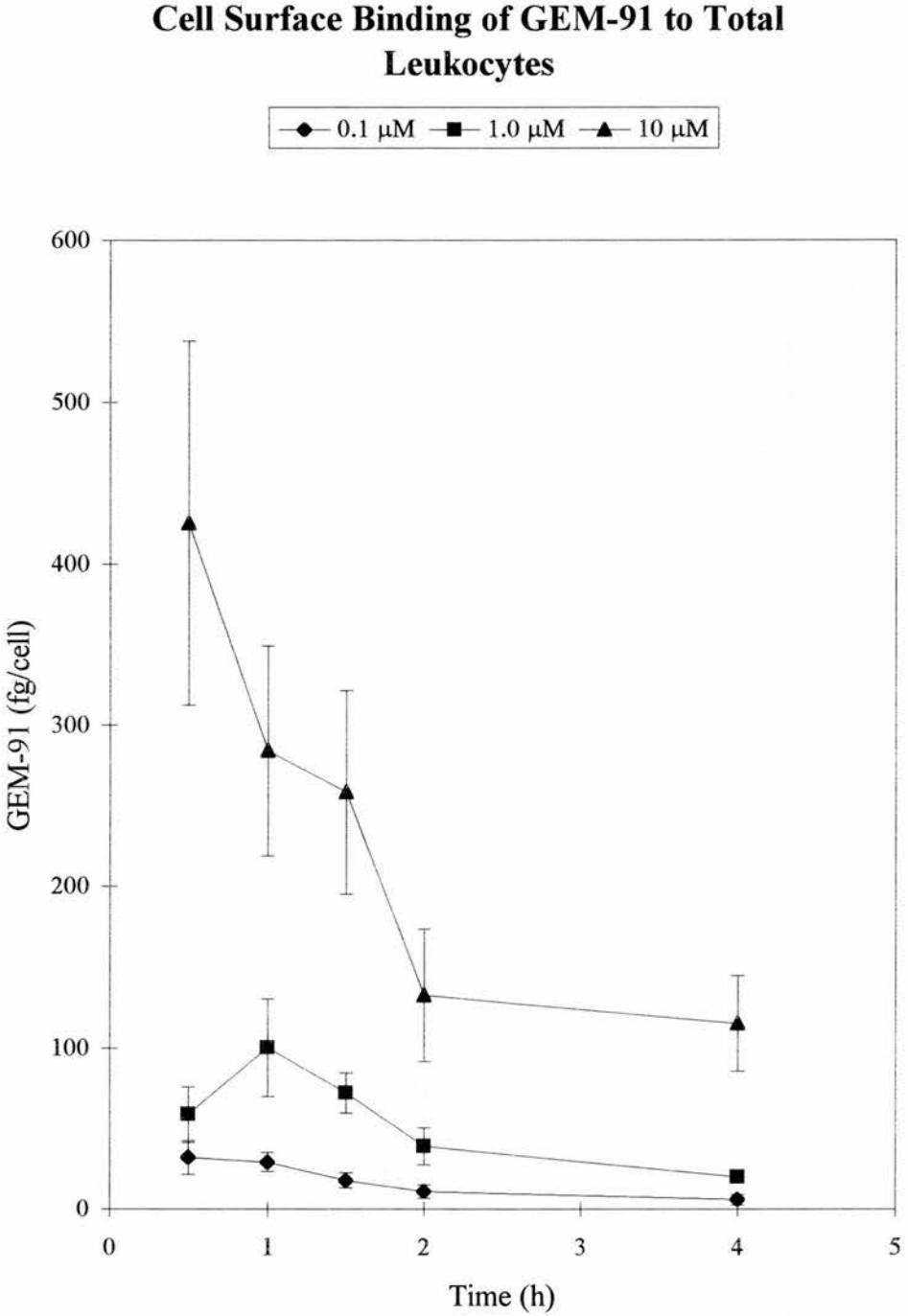


FIGURE 3.5 Cell Surface Binding of GEM-91 to Total Peripheral Blood Leukocytes. PBLs were incubated with 3 different concentrations of biotin-labelled GEM-91 for 4h. Surface bound ODN was detected by addition of PE-labelled streptavadin, which cannot penetrate cells and so binds only to cell-surface associated ODN. It can be seen that after a 30 min incubation with 10μM GEM-91 PBMCs bound approximately 425fg GEM-91/cell. This concentration gradually decreased over the 4 hour incubation period. Points are the mean of 3 separate experiments +/- sem.

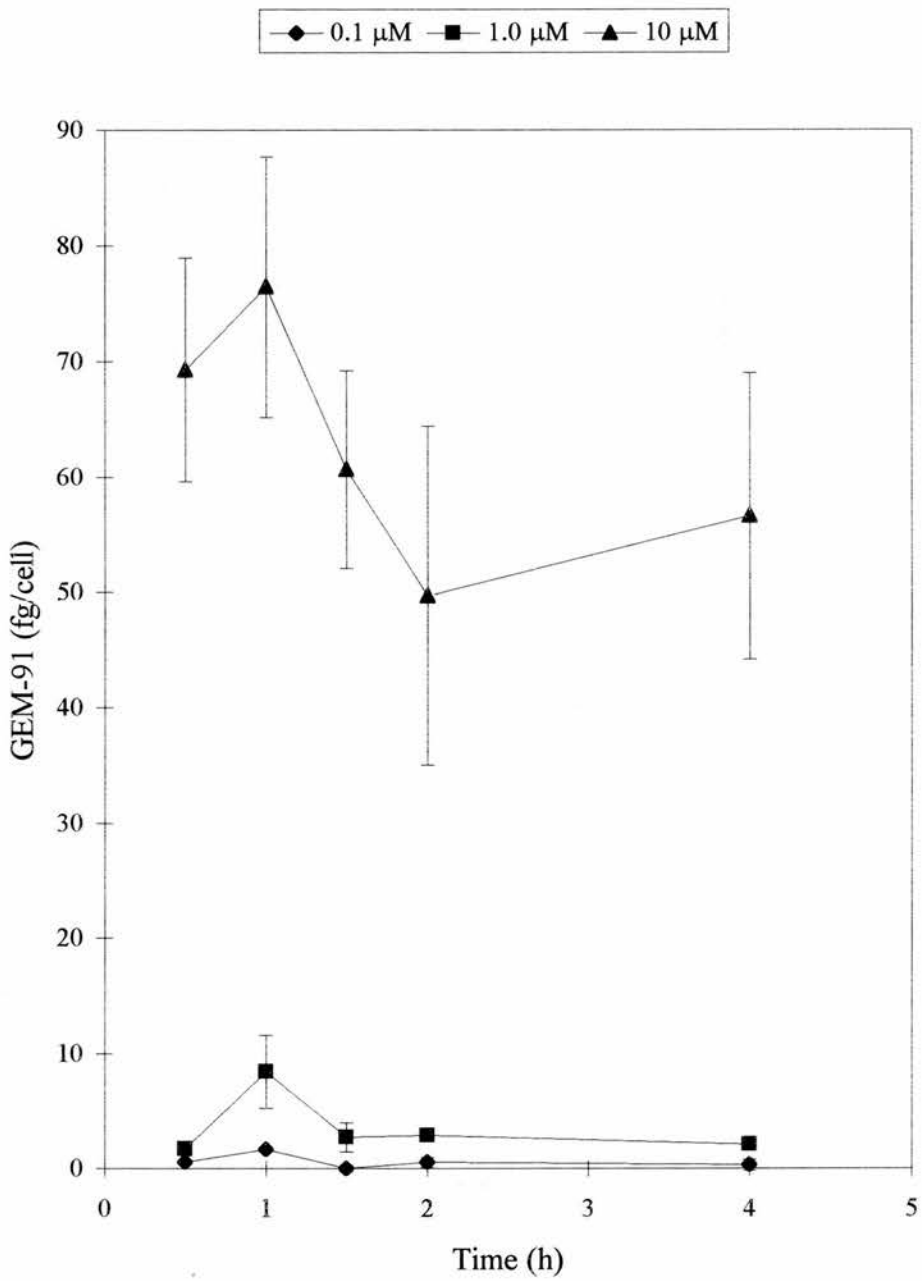


FIGURE 3.6 Cell Surface Binding of GEM-91 to Lymphocytes. Cells were incubated with 3 different concentrations of biotin-labelled GEM-91 for 4 hours. Cell surface bound GEM-91 was detected by the addition of PE-labelled streptavadin which cannot penetrate cells and so binds only to cell surface associated ODN. After a 1 hour incubation with 10μM GEM-91 lymphocytes bound approximately 75fg GEM-91/cell. There was no measurable decrease in extracellular associated ODN over the 4 hour incubation period. Points are the mean of 3 separate experiments +/- sem.

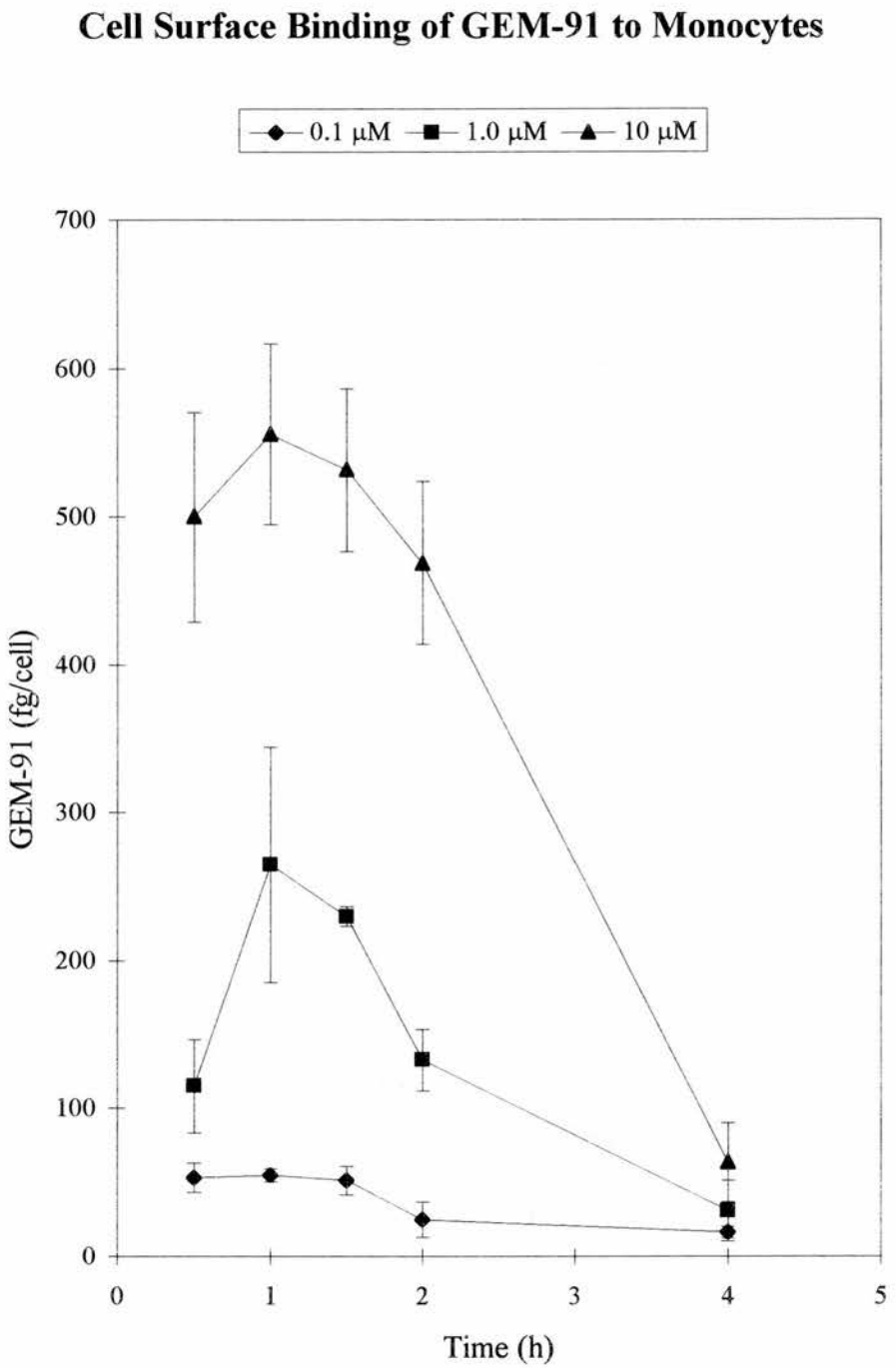


FIGURE 3.7 Cell Surface Binding of GEM-91 to Monocytes. Cells were incubated with 3 different concentrations of biotin-labelled GEM-91 for 4 hours. Cell surface bound ODN was detected by incubation with PE-labelled streptavadin which cannot penetrate cells and so binds only to cell suface bound ODN. It can be seen that after 30 minutes in culture with 10μM GEM-91 monocytes bound approximately 500fg GEM-91/cell. This dropped dramatically to 60fg/cell after 4 hours despite the continued presence of GEM-91 in the cultures. Points are the mean of 3 separate experiments +/- sem.

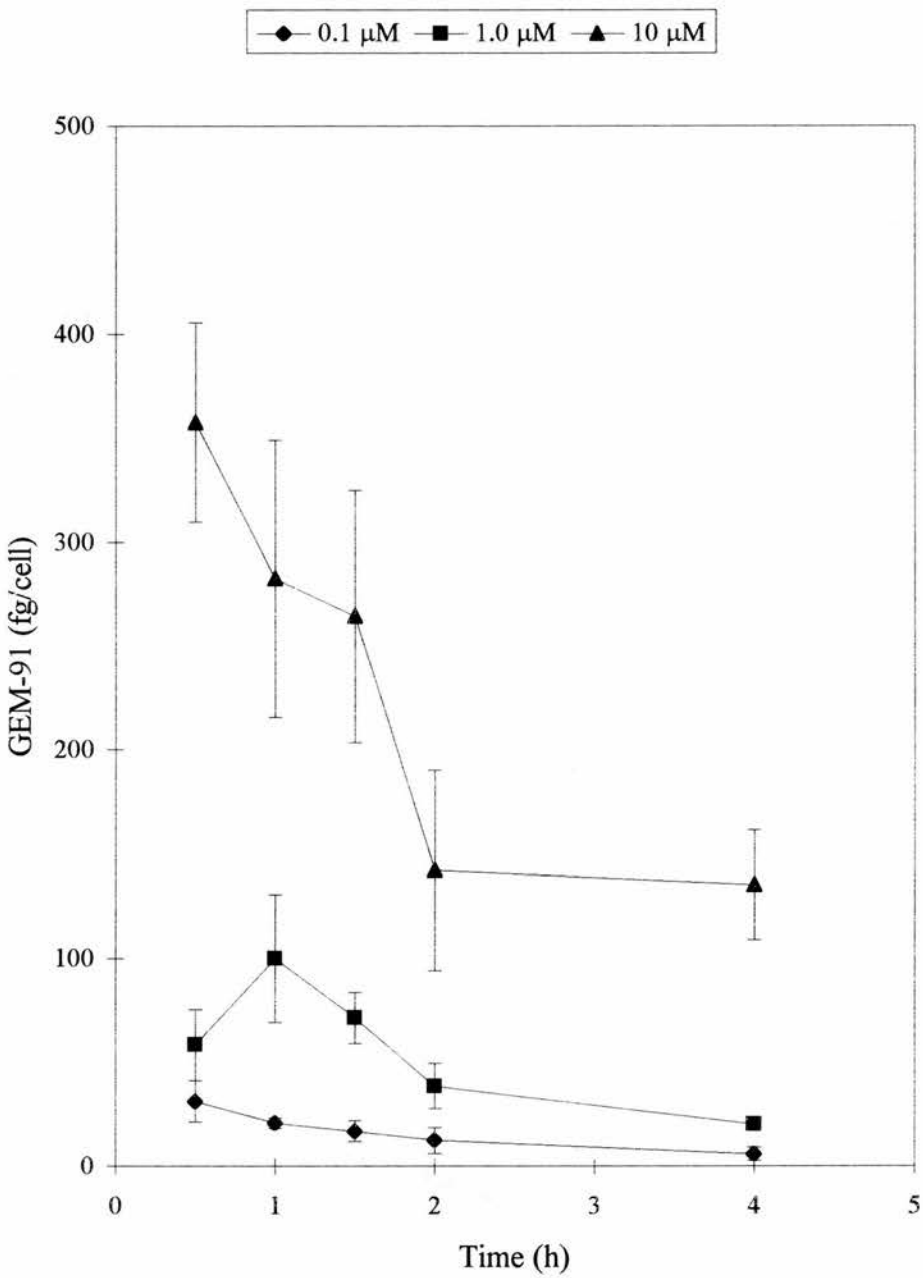


FIGURE 3.8 Cell Surface Binding of GEM-91 to Neutrophils. Cells were incubated with 3 concentrations of biotin-labelled GEM-91 for 4 hours. Surface-bound ODN was detected by the addition of PE-labelled streptavadin which cannot penetrate cells and so detects only surface bound ODN. After 30 minutes in the presence of 10μM GEM-91 neutrophil cell surface bound ODN had reached approx. 350fg/cell decreasing to 135fg/cell after 4 hours. Points are the mean of 3 separate experiments +/- sem.

3.3.2 Intracellular Uptake of GEM-91 into Peripheral Blood Leukocytes

3.3.2.1 Total Leukocyte Population

Intracellular uptake of GEM-91 was dose- and time- dependent, and was not saturated at concentrations of GEM-91 up to 10μM for 4 hours. By 4 hours of incubation, intracellular concentrations had reached levels equivalent to extracellular concentrations achieved in the first hour (Fig. 3.7).

3.3.2.2 Lymphocytes

At the lowest concentration of GEM-91 (0.1μM), no detectable level of uptake of the ODN into lymphocytes was measurable at timepoints < 4 hours. At this dose, the level of GEM-91 measured after 4 hours was 3.7fg GEM-91/cell. At the intermediate concentration of GEM-91 (1.0μM), there was no change in the ODN uptake over the first 4 hours of culture. The level of GEM-91 measured between 30 minutes and 4 hours in culture remained constant at approximately 15fg GEM-91/cell. At the highest concentration of GEM-91 (10μM), the peak value of ODN uptake was approximately 94fg GEM-91/cell after 2 hours (Fig 3.8).

3.3.2.3 Monocytes

As with the extracellular studies, monocytes exhibited the highest levels of GEM-91 uptake, with approximately 2- and 4- fold higher levels of GEM-91 than that measured in neutrophils and lymphocytes respectively. At the highest dose, (10μM), the mean concentration of GEM-91 within monocytes was approximately 200fg GEM-91/cell after 0.5 hours in culture. This concentration increased to approximately 800fg GEM-91/cell after 4 hours (Fig 3.9).

3.3.2.4 Neutrophils

Neutrophils also showed a high level of intracellular GEM-91. At the highest dose (10μM), the mean concentration of GEM-91 within neutrophils was approximately 130fg GEM-91/cell after 0.5 hours in culture. This concentration increased to approximately 460fg GEM-91/cell after 4 hours. As in the extracellular studies, this increase was less marked than that measured for monocytes (Fig 3.10).

Raw data for intracellular binding in all cell types can be found in Appendix B2.

Finally, it should be noted that the uptakes observed for the various cell populations add up to the total leukocyte value observed after adjustment for differential cell content.

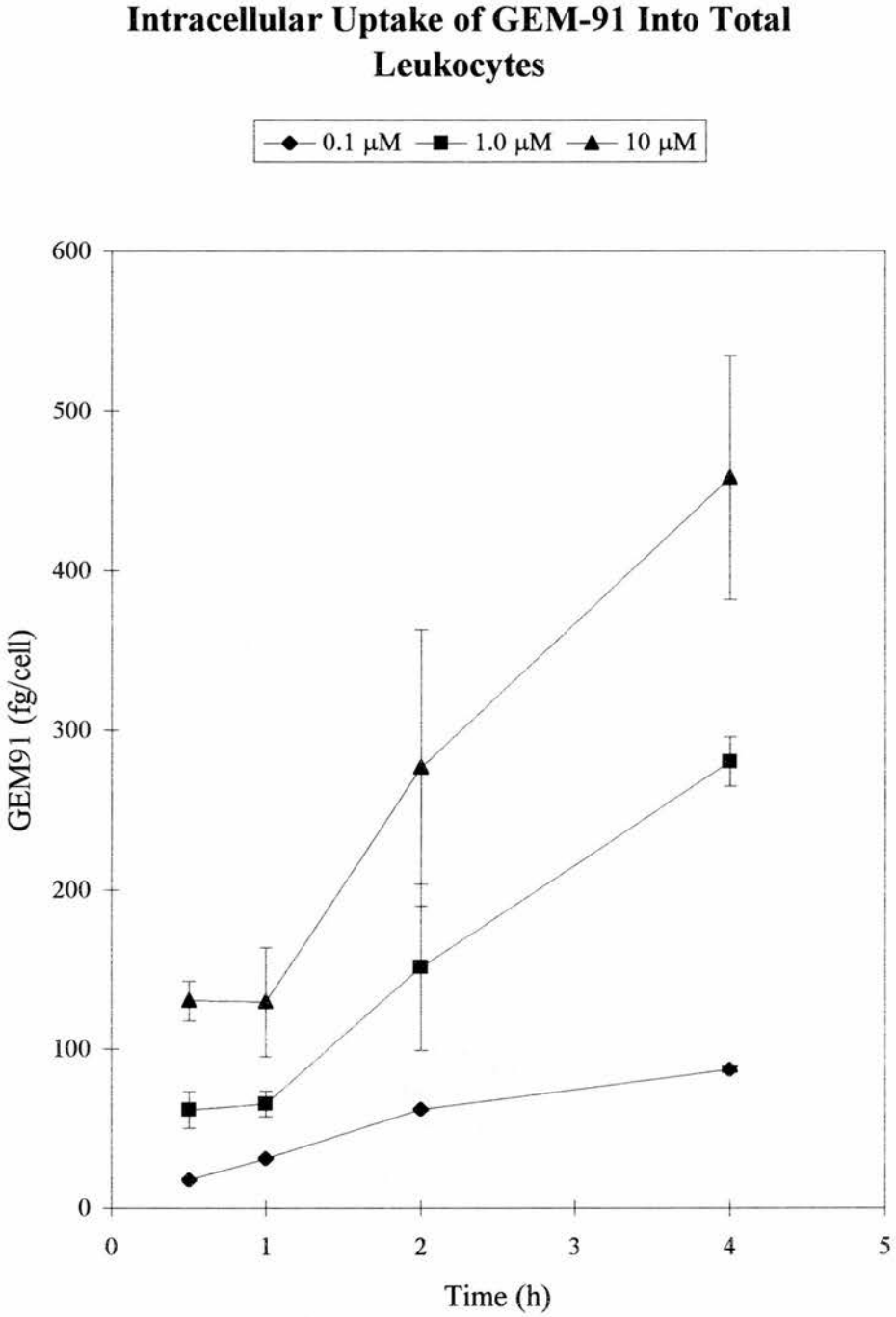


FIGURE 3.9 Intracellular Uptake of GEM-91 into Total Leukocytes. PBLs were incubated with 3 concentrations of FAM-labelled GEM-91 for 4 hours after which they were treated with 0.25% propidium iodide (PI). This compund quenched the cell surface FAM-labeled GEM-91 enabling the intracellular GEM-91 to be measured directly. ODN uptake was time and dose dependent, and after 4h in culture intracellular concentrations reached levels equivalent to extracellular concentrations achieved in the first hour. Points are the mean of 3 separate experiments +/- sem.

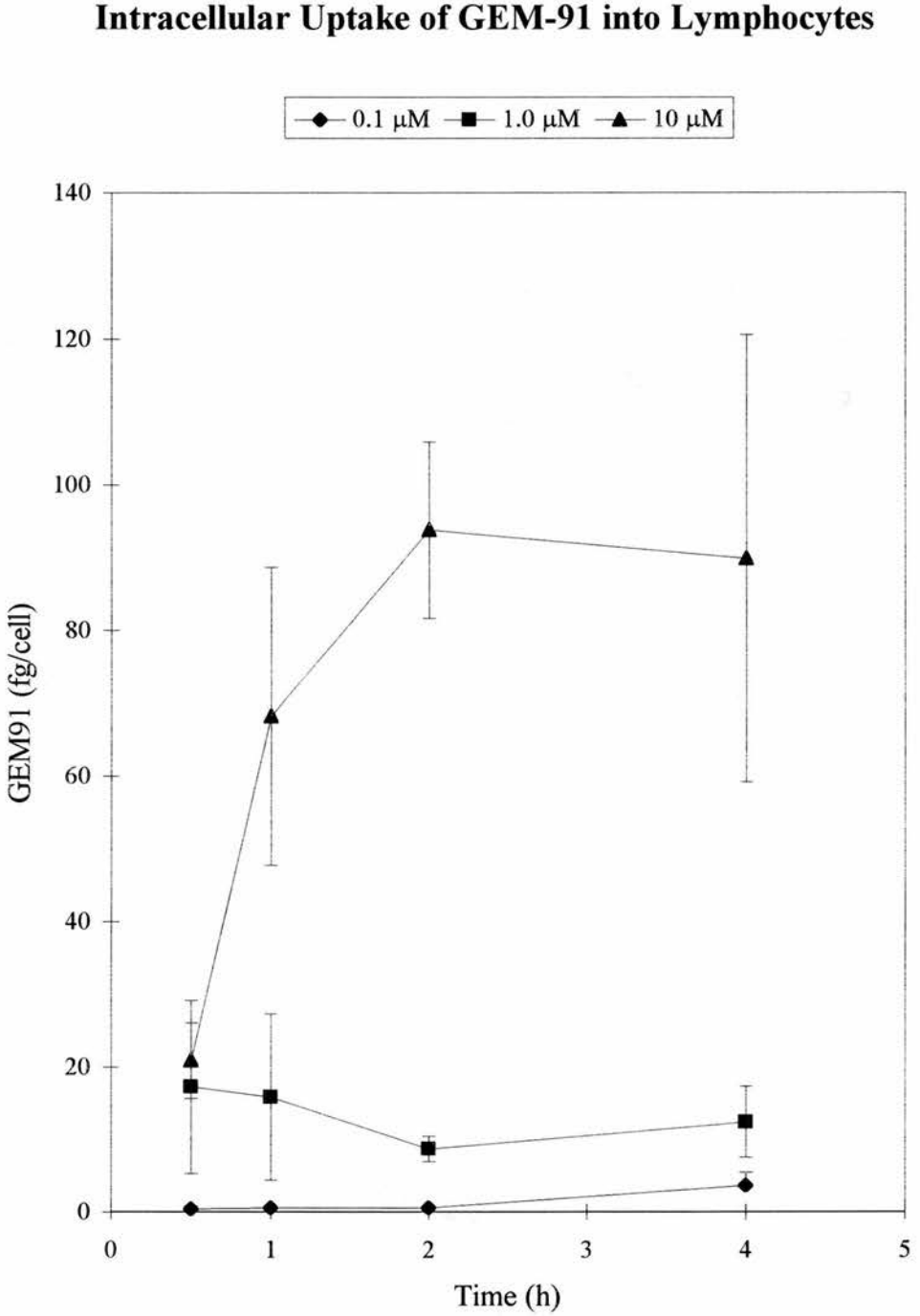


FIGURE 3.10 Intracellular Uptake of GEM-91 into Lymphocytes. Cells were incubated with 3 concentrations of FAM-labelled GEM-91 for 4 hours after which they were treated with 0.25% PI to quench the cell surface FAM-labeled GEM-91 enabling the intracellular GEM-91 to be measured directly. At the lower doses of ODN tested there was very little internalization of ODN. After 2h in culture with 10μM GEM-91 lymphocyes internalized approx. 94fg/cell . Points are the mean of 3 separate experiments +/- sem.

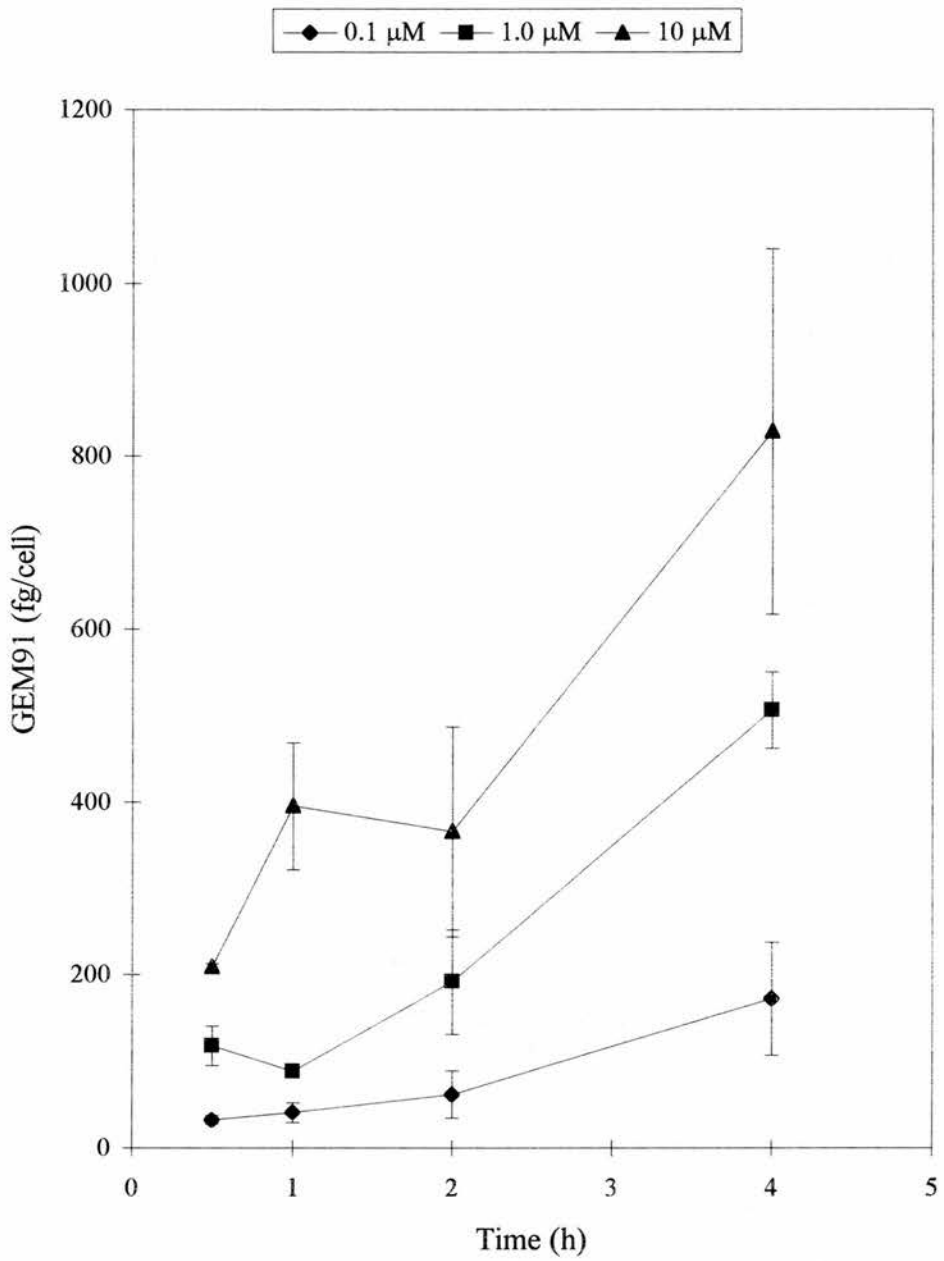


FIGURE 3.11 Intracellular Uptake of GEM-91 into Monocytes. Cells were incubated with 3 concentrations of FAM-labelled GEM-91 for 4 hours after which they were treated with 0.25% PI to quench the cell surface FAM-labelled GEM-91 enabling the intracellular GEM-91 to be measured directly. After 30mins in culture with 10μM GEM-91 monocytes internalized approx. 200fg/cell, increasing to approx. 800fg/cell after 4h in culture. The increase in intracellular concentrations over 4h were concommitant with the decrease in extracellular binding over this time period. Points are the mean of 3 separate experiments +/- sem.

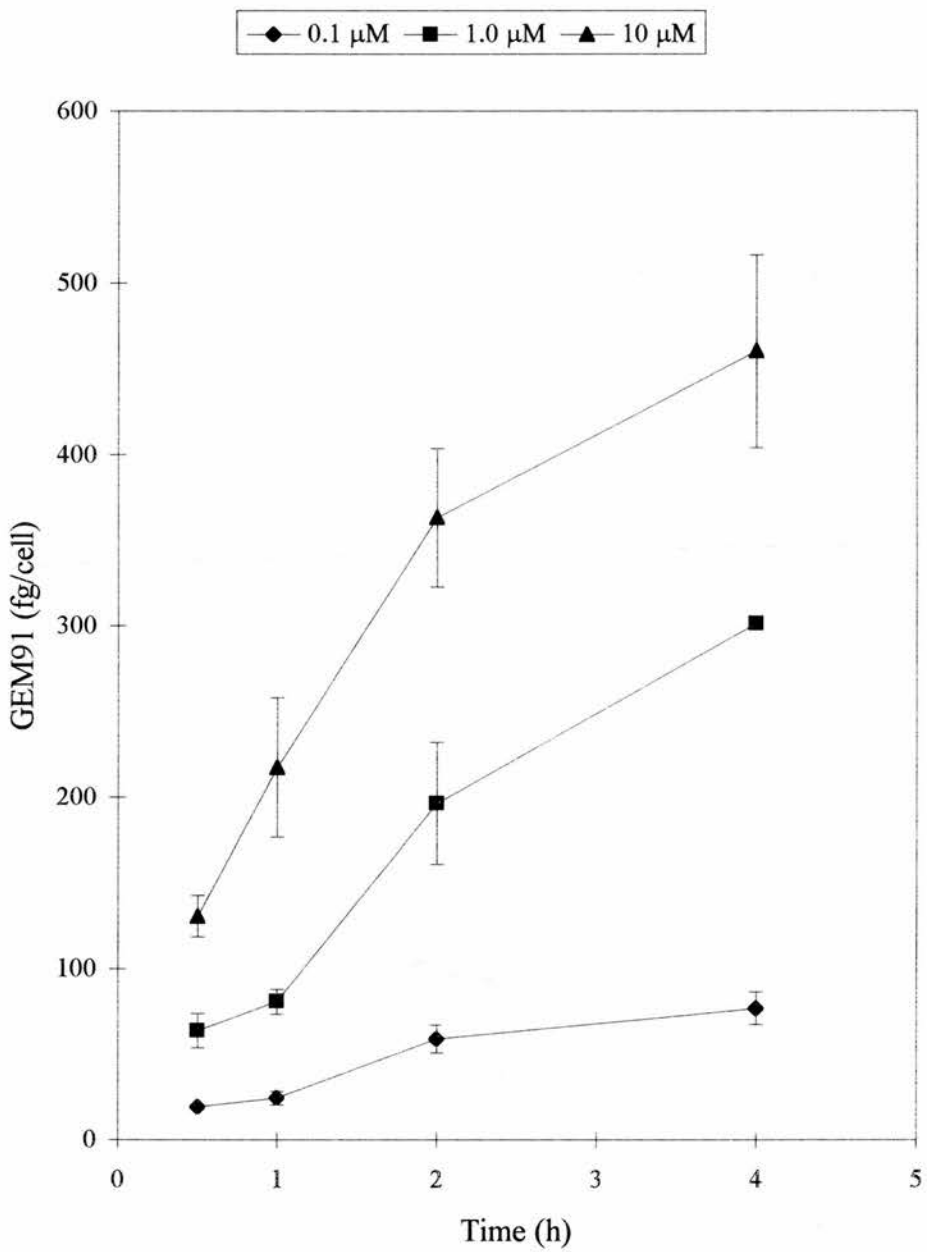


FIGURE 3.12 Intracellular Uptake of GEM-91 into Neutrophils. Cells were incubated with 3 concentrations of FAM-labelled GEM-91 for 4 hours after which they were treated with 0.25% PI to quench the cell surface FAM-labelled GEM-91. After 30mins in culture with 10μM GEM-91 monocytes internalized approx. 130fg/cell, increasing to approx. 460fg/cell after 4h in culture. The increase in intracellular concentrations over 4h were concommitant with the decrease in extracellular binding over this time period. Points are the mean of 3 separate experiments +/- sem.

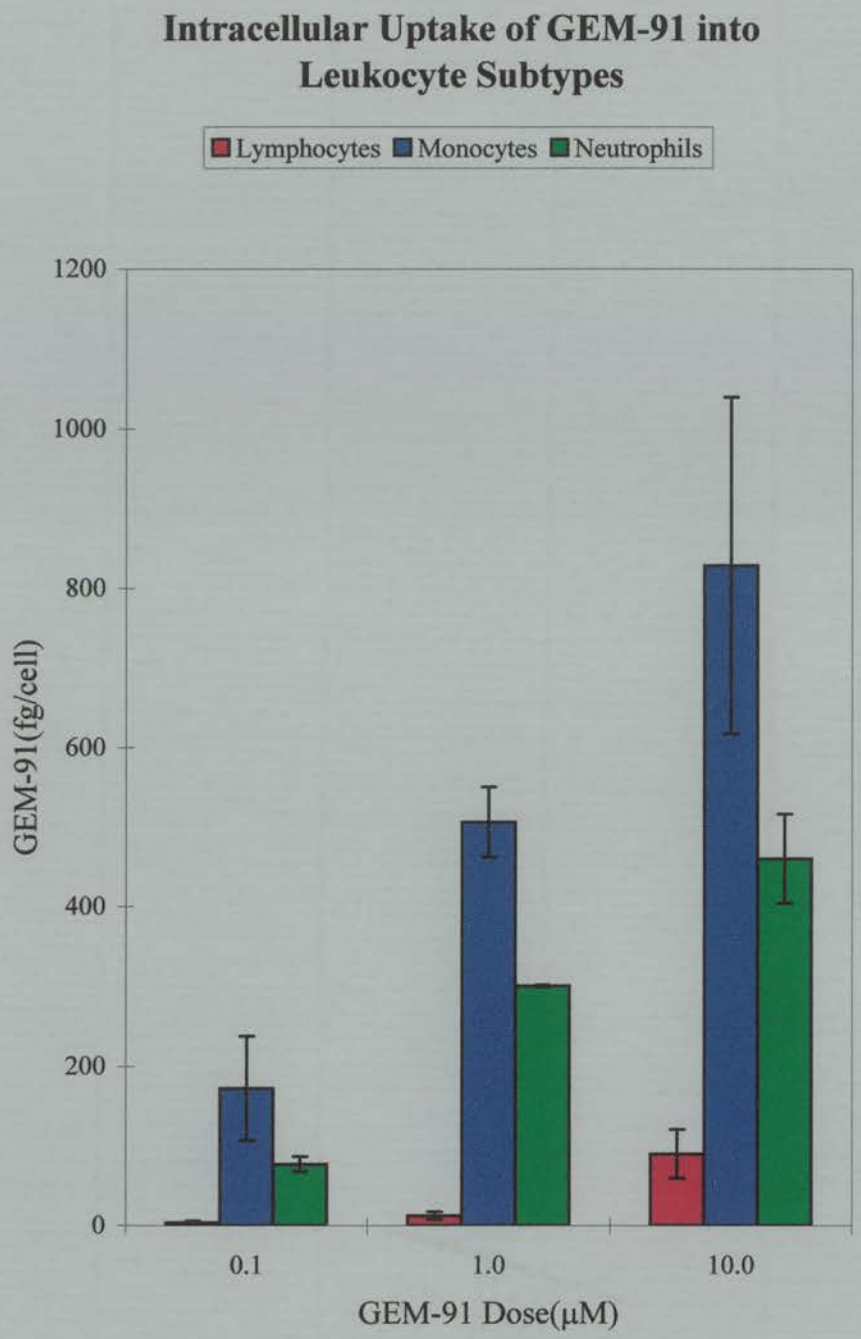


FIGURE 3.13 Intracellular Uptake of GEM-91 into Peripheral Blood Leukocyte Subtypes.
Leukocytes were incubated with 3 concentrations of FAM-labelled GEM-91 for 4h after which time they were treated with 0.25% PI. This quenched the cell surface bound fluorescence enabling intracellular fluorescence to be directly measured. It can bee seen that at all the doses of GEM-91 tested monocytes internalized more ODN than either lymphoctes or neutrophils.

3.4 Discussion

The object of the experiments described in this chapter was to investigate the uptake characteristics of the naked, 25 base phosphorothioate ODN GEM-91 into peripheral blood leukocyte subtypes in an attempt to determine the suitability of the monocyte as a target for antisense therapy. Large differences between cell types have been reported regarding oligonucleotide incorporation. Primary keratinocytes, for example, have been shown to display nuclear accumulation of naked phosphorothioate ODNs and specific inhibition of ICAM-1 expression (Nestle *et al.*, 1994). In the same study, however, endothelial cells smooth muscle cells and fibroblasts did not show ODN accumulation in nuclei without the use of a lipid carrier. In contrast to cell lines, freshly isolated PBLs represent a more heterogeneous cell population not only with respect to cell type but also in terms of the state of cell activation and differentiation, and so provide a more physiological environment in which to investigate the uptake of ODN into monocytes. The data presented in this chapter clearly demonstrated a dose- and time- dependent uptake of ODN into PBLs in the concentration range 0.1-10μM over a 4 hour period. It was apparent that this uptake comprised extracellular and intracellular components which were both dose dependent, and that there was heterogeneous uptake between the different PBL subtypes. In the total cell population, extracellular binding as measured by biotin-labelled GEM-91 detected by PE-conjugated streptavadin, was found to be dose dependent and non saturating in the range of concentrations tested (0.1-10μM). The highest levels of extracellular association were detected at the earlier timepoints with significant decreases being observed over time. After 30 minutes in the presence of 10μM GEM-91 the mean concentration of ODN on the cell surface was 425 ± 112 fg/cell. This concentration decreased to 115 ± 30 fg/cell over the 4 hour culture period (fig 3.3). This decrease in extracellular binding was associated with a concomitant increase in intracellular GEM-91 levels as measured by FAM-labelled GEM-91 in conjunction with PI. The intracellular GEM-91 levels increased from 130 ± 12.4 fg/cell after 30 minutes in culture to 458 ± 66.5 fg/cell after 4 hours These intracellular levels were approximately equal to the extracellular levels achieved in the

first hour. This pattern of ODN association strongly suggests that there is cellular internalization of the ODN over the 4 hour period. The efficiency of this internalization of GEM-91 varied between the PBL subtypes tested. After 2 hours in culture at ODN doses of 0.1, 1 and 10μM the intracellular levels of GEM-91 in monocytes were 100, 23 and 4 fold higher respectively than in lymphocytes. At this timepoint, the intracellular ODN levels were similar in monocytes and in neutrophils at all doses tested. However, after 4 hours in culture with the same three concentrations of ODN, monocytes internalised approximately 45 times more ODN than lymphocytes at the two lower doses and 10 times more at the 10μM dose. Furthermore, after 4 hours in culture at all doses tested, monocytes internalized twice as much ODN as neutrophils. These results suggest that monocytes internalise ODN preferentially over lymphocytes and neutrophils after a 4 hour incubation Indeed at the highest dose of ODN administered (10μM), monocytes contained 828fgODN/cell which equates to $5x10^7$ molecules of DNA. Before 1997, only 4 studies had examined uptake of ODNs in human PBLs and only one of these described spontaneous uptake in primary monocytes. Pirrucello *et al.*, 1994 investigated uptake of a 27 base ODN at a concentration of 4μM into primary cultured monocytes by confocal microscopy. They observed that after a 2 hour incubation, 75-85% of monocytes and 42-61% of lymphocytes were ODN positive. No information was given as to the actual quantity of ODN internalized by these cell types. Three other studies have described uptake of ODNs in human PBLs. Marti *et al.*, 1992 have stated that incorporation of ODNs in PBMCs is one tenth of that in H9 cells (a human T-lymphocyte cell line). No specification for mononuclear cell subtypes was made in this study. Another group reported the opposite namely that uptake of ODNs in PBMCs is more than two times higher than uptake in H9 cells (Iversen *et al.*, 1992). However, again mononuclear cell subpopulations were not examined in this study. Zhao *et al.*, 1996 compared uptake of three FITC labelled ODNs, two 20mers and one 25mer in human PBMCs, bone marrow cells and a human promyelocytic leukaemia cell line. The three ODNs were used interchangeably as no differences in their uptake was seen. It was shown that uptake was heterogeneous between different cell types in normal PBLs and in bone marrow cells, in that B-lymphocytes

took up more than T-lymphocytes and myleoid/macrophages took up more than either lymphocyte subtype. Interestingly, in contrast to my observations, neutrophils exhibited a very low ODN uptake. In addition, it was observed that human leukaemic cells took up more ODN than normal cells from the same patient. It was speculated that this was due to the fact that leukaemic cells are more actively dividing than normal cells. Zhao *et al.*, 1996 also showed that ODN uptake is closely related to the cell cycle in that activated cells take up more ODN than non-active cells. More recently, during the course of my studies, Hartmann *et al.*, 1998 have examined spontaneous uptake of a FITC labelled 18 base phosphorothioate ODN by flow cytometry and fluorescence microscopy. They have shown that in the presence of 1μM naked ODN incubated for 2 hours 100% of monocytes and B- lymphocytes incorporated ODN compared with only 12% of natural killer cells and 1% of T-lymphocytes. They observed that after 2 hours at a concentration of 1μM monocytes internalize 4 times more ODN than lymphocytes. This is in contrast to the current study where after a 2 hour incubation with 1μM ODN monocytes internalized approximately 20 times more ODN than lymphocytes in the absence of any cationic lipids. At the higher dose of 10μM ODN lymphocytes managed to internalize more ODN, but monocytes still contained 4 times more than them. Hartman *et al.*, 1998 did not investigate ODN uptake into neutrophils.

Experiments have been performed by groups aimed at tracking the progress of ODNs through cells. These experiments have been carried out in haematopoietic cell lines and not in PBLs. It has been reported that in HL-60 human promyelocytic leukaemia cells, ODN appeared to localize in endosomes in which they are acidified and undergo efflux relatively slowly (Tonkinson *et al.*, 1994). However, in K562 human chronic leukaemia cells, once internalized, ODNs move from the endosomal compartment into the cytoplasm and from there into the nucleus. Hartmann *et al.*, 1998 have investigated ODN uptake into isolated primary monocytes and have shown that complex formation of the ODN with the cationic lipid lipofectin, enhanced uptake and improved intracellular distribution in monocytes 5-10 fold. This effect, however, depended on the ratio of lipid to ODN and was effective only in a narrow concentration range, namely a 1:1 ratio of ODN to lipofectin, as larger quantities of

lipids are cytotoxic. For this reason, cationic lipid mediated ODN transfer is not appropriate for systemic administration of ODNs but may be effective for locally administered ODN where lower concentrations are required. In agreement with Hartmann *et al.*, we have shown that monocytes take up more ODN than lymphocytes. However, in the current study the differences are more striking, monocytes internalizing more ODN compared with lymphocytes at 1μM and 10μM after 2 hours and 4 hours in culture suggesting that sufficient monocyte loading with ODN may be achieved at lower doses than previously thought and without the need for cationic lipids. This suggests that monocytes may be good target cells for antisense therapy. Hartmann *et al.*, isolated peripheral blood mononuclear cells by ficoll hypaque centrifugation which removed neutrophils, and resuspended their cells in RPMI 1640 culture medium. In our study leukocytes were isolated by red-cell lysis followed by resuspension in QBSF culture medium. It is possible that the presence of neutrophils and/or platelets in the cell suspension may have increased the activation state of monocytes through cell adherence causing them to take up more ODN, either by increasing the density of cell surface receptors or by promoting the differentiation of monocytes into the more phagocytotic macrophages. However, to fully investigate the intracellular distribution of ODN within human PBLs to determine whether or not the ODN reaches its mRNA target would require powerful microscopy carried out on freshly isolated cells. It has been demonstrated by Beltinger *et al.*, 1992 that at lower concentrations (<1μM) ODNs enter cells mainly by receptor mediated endocytosis whereas at higher concentrations fluid phase endocytosis becomes more obvious. It is tempting to speculate that the higher levels of extracellular binding and internalization of ODN seen in monocytes and neutrophils represents the presence of higher levels of DNA binding proteins. This being so, it may be postulated that the decrease observed in extracellular association of GEM-91 over time despite the continued presence of ODN in the culture medium may be due to a down regulation in these binding proteins or receptors. This may theoretically occur as a result of DNA binding and subsequent receptor internalization or alternatively it may represent a change in cell phenotype during culture with ODNs. It has been shown by many groups that certain cell surface receptors are involved in receptor mediated endocytosis of naked DNA

into cells including CD11b/CD18 (Mac-1) (Benimetskya *et al.*, 1997), which is expressed primarily on monocytes and neutrophils but not on lymphocytes. Therefore, it is possible that ODN may be entering monocytes and neutrophils preferentially by receptor mediated endocytosis either via a specific receptor or by a more generalised receptor mechanism.

In summary, I have shown that ODNs enter peripheral blood leukocytes in the naked state without the use of a carrier molecule and that this uptake is dose and time dependent. Furthermore, it has been shown that monocytes internalize more ODN than either neutrophils or lymphocytes. Therefore, it is clear that monocytes represent a potential target for ODN therapy. However, it is not clear from these studies whether ODN is bioavailable intracellularly to act as an efficient antisense molecule.

CHAPTER 4

INVESTIGATION OF PUTATIVE ANTISENSE RECEPTORS

4.1 Introduction

I have previously examined the uptake kinetics of a 25 base ODN into PBMC and concluded that the uptake is both dose and time dependent. The changes in the extracellular association of the oligo with monocytes and neutrophils were measured. There was an initial high extracellular association of ODN and, despite the continued presence of ODN in the culture medium a decrease in cell surface bound oligo was observed over time. In addition, there was a concomitant increase in intracellular ODN as the extracellular concentration decreased. From these observations it may be speculated that the higher levels of ODN on the cell surface of monocytes and neutrophils represents higher levels of DNA binding proteins. If this were the case it may be concluded that the decrease observed in the extracellular association of the oligo over time despite the continued presence of ODN may represent a down regulation of these binding proteins or 'receptors'. This may theoretically occur either as a consequence of DNA binding and subsequent internalisation as in receptor mediated endocytosis. Alternatively, it may be a consequence of changes in cell phenotype during culture.

4.1.1 Mechanisms of Oligonucleotide Uptake

Uptake of DNA appears to be a natural phenomenon that has been postulated to represent a nucleic acid salvage mechanism for material excreted by apoptotic cells (Bennett, 1993). A number of laboratories have examined ODN uptake using either native, methylphosphonate or phosphorothioate DNA (Yakubov *et al.*, 1989; Loke *et al.*, 1989; Geselowitz and Neckers 1992; Gao *et al.*, 1992; Beltinger *et al.*, 1995). Methylphosphonate molecules are uncharged molecules that have been reported to enter cells via passive diffusion (Miller, 1991). In contrast, native phosphodiester and phosphorothioate molecules are large polyanionic molecules which cannot easily passively cross cellular membranes. Therefore, it appears that ODN uptake is an active process dependent on concentration, energy and temperature. This appears to

be accomplished primarily through a combination of adsorptive endocytosis and fluid phase endocytosis which may be triggered in part by the binding of the ODN to receptor-like proteins present on the surface of a wide variety of cells (Gao *et al.*, 1993; Beltinger *et al.*, 1995). Studies by Beltinger *et al.*, in K562 cells (human leukaemia cell line) have demonstrated that the uptake mechanism is at least partially concentration dependent, and that below a concentration of 1μM, uptake of phosphorothioate ODNs is predominantly via a receptor-like mechanism, whilst at extracellular concentrations higher than this, adsorptive and fluid phase endocytosis predominates. Furthermore, using electron microscopy, Beltinger *et al.*, 1995 have detected ODN in clathrin coated pits on the cytoplasmic membrane, within endosomes and lysosomes as well as free in the cytoplasm. In addition, the ODN uptake can be inhibited by an unlabelled competing ODN. This work strongly suggests that a receptor mediated mechanism is involved in ODN uptake, and that, in K562 cells at least, naked ODN is capable of reaching the cytoplasm and indeed the nucleus and thus is likely to be available to bind to target mRNA.

4.1.2 Putative Oligonucleotide Cellular Receptors

The identity of cell membrane proteins that bind ODNs is at present unclear. Proteins with molecular weights of 75-90 kDa have been found to bind ODNs in HL60 cells and have been termed nucleic acid binding protein-1 (NABR-1) (Yakubov *et al.*, 1989). Loke et al, 1989 have, using affinity chromatography, isolated an 80 kDa surface protein that appeared to be responsible for nucleic acid transport. Subsequently, Beltinger *et al.*, 1995 investigated the binding of biotinylated phosphorothioate ODNs to the cell membranes of K562 cells and found ODN binding proteins of 137-147, and 79-85 kDa, as well as species of lower molecular mass. In preparations of purified renal tubular brush border membranes, Rappaport *et al.*, 1995 identified a phosphorothioate ODN-binding protein of 97 kDa. However, none of these putative binding proteins was further characterised. Guvakova *et al.*, 1995 have shown that phosphorothioate ODNs can bind to a large number of heparin binding proteins, including basic and acidic fibroblast growth factor (bFGF and aFGF),

platelet derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), as well as to the VEGF receptor. The affinity of phosphorothioate ODNs for bFGF can be high (30nM), is dependent on ODN length and, to some extent, ODN sequence as well. Kimura *et al.*, 1994 reported that ligands for scavenger receptors blocked ODN induction of interferon production on murine natural killer (NK) cells and proposed that scavenger receptors bound ODNs.

In 1997, Benimetskya *et al.*, demonstrated using HL60 cells, the involvement of the Mac-1(CD11b/CD18) receptor in ODN uptake into cells. Mac-1 is a member of a family of leukocyte integrins that are involved in leukocyte adhesion and migration, and that are involved in the binding of polymorphonuclear leukocytes (PMN) to endothelial cells and the extracellular matrix. Mac-1 is also found on monocytes, macrophages and NK cells. Mac-1 is a heparin binding protein (Coombe *et al.*, 1994; Diamond *et al.*, 1995) and is a cell surface receptor for a variety of ligands including fibrinogen (Loike *et al.*, 1991; Wright *et al.*, 1988), C3bi (the cleaved third component of complement) (Wright *et al.*, 1983), intercellular adhesion molecule-1 (Diamond *et al.*, 1991), factor X (Alteri *et al.*, 1988) and fibrin (Loike *et al.*, 1995). Benimetskya *et al.*, have demonstrated that both phosphodiester and phosphorothioate ODNs bind to both the αM and β2 chains of Mac-1 on polymorphonuclear leukocytes. They have also shown that there is a correlation between this binding and the rate of intracellular ODN internalization and that ODN binding can be inhibited by heparin and fibrinogen.

In the light of these experiments, we have investigated the effect of GEM-91 on monocyte cell surface Mac-1 and CD14 levels (another receptor found in abundance on monocytes which has not been implicated in ODN binding). We have shown that monocytes internalize ODN more efficiently than any other PBMC, and since monocytes express high levels of both Mac-1 and CD14, we have investigated the relative contribution of these receptors in binding GEM-91.

4.2 Materials and Methods

4.2.1 Blood Cell Collection and Isolation

Peripheral venous blood was collected and leukocytes isolated as previously described.

4.2.2 Leukocyte Incubations

Leukocytes were adjusted to a concentration of $1x10^7$ cells per ml in QBSF media. Aliquots (2ml) were incubated in sterile polypropylene tubes at 37°C, 95% air 5% CO_2 for 0-4 hours. Cells were either treated with 5μM GEM-91 labelled with FAM, 5μM GEM-91 unlabelled, or sterile distilled water as a control. The 5μM dose of GEM-91was chosen to conserve costs as it represented a compromise between the highest (10μM) and the intermediate (1μM) doses tested in chapter 3.

4.2.3 Preparation of Cells for Flow Cytometry

At timepoints of 0.5, 1, 2, 4 hours ,100ul aliquots of cells ($1x10^6$) were removed and directly immunostained for CD14 and Mac-1(CD11b/CD18) as described in chapter 2. To stain for Mac-1(CD11b/CD18) 10μl of 70μg/ml stock solution of monoclonal anti-human CD11b clone 44 conjugated with quantum red fluorochrome (Sigma Chemical Co.) or a negative isotype control antibody (mouse IgG1 conjugated with quantum red, Sigma Chemical Co.) was added to 100μl cells.

To detect the CD14 antigen, which is a specific monocyte cell surface marker, 100μl cell aliquots were stained with monoclonal anti-human CD14 clone UHM-1 conjugated with R-PE (Sigma Chemical Co.) or with the isotype control antibody (IgG1 conjugated with R-PE) as above.

4.2.4 Flow Cytometric Analysis

Flow cytometry was carried out immediately, using the FL3 channel to detect CD11b positive cells and the FL-2 channel to detect CD14 positive cells. An average of 50000 cells were collected from each sample, typically containing 1500-2500 monocytes. Cell types were identified by virtue of their forward and side scatter characteristics, cells in the monocyte gate being typically 95% CD14 positive when no oligo was added. The fluorescence channel gains were zeroed using the cells stained with the negative isotype controls so that all cells fell in the first log decade. The geometric mean fluorescence of the cells stained with the test antibody was recorded. The geometric mean fluorescence of the cells after treatment with the ODN was expressed as a percentage of the fluorescence of the untreated cells.

4.2.5 Statistical Analysis

All analyses were done on raw data and were carried out using a repeated measures analysis of variance followed by a Bonferroni multiple comparisons post test. Where data could not be matched due to varying numbers in each group, a one way analysis of variance was carried out on the mean values followed also by a Bonferroni multiple comparisons post test.

4.3 Results

4.3.1 Effect of Culture Conditions on Monocyte Cell Surface Mac-1(CD11b) and CD14 Levels

A 100% value was assigned to the CD11b and CD14 levels at baseline i.e. t=0.
After 0.5 hour in culture alone, the cell surface levels of Mac-1 decreased to 83 ± 5% of baseline at 1h ($p<0.01$), 82 ± 6% of baseline at 2h ($p<0.001$), 73 ± 2 of baseline at 4h ($p<0.001$) (Fig 4.1a).
The cell surface CD14 levels did not significantly decrease over the 4 hour culture period compared with the t=0 baseline (Fig 4.1b).

4.3.2 Effect of GEM-91 on Monocyte Cell Surface Mac-1/(CD11b/CD18)

After 0.5 hours in culture with 5μM GEM-91, the cell surface levels of Mac-1 were already significantly decreased with respect to the untreated cells at the same timepoint and they continued to decrease in a time dependent manner over the untreated cells at 1, 2 and 4 hours in culture. At t=0.5 the oligo treated cells had CD11b levels 51 ± 4% of baseline, $p<0.001$, at t=1 38 ± 3 % of baseline $p<0.001$, at t=2, 31 ± 2.5% of baseline $p<0.001$ and at t=4, 24 ± 2% of baseline $p<0.001$ (Fig 4.1a).

4.3.3 Effect of GEM-91 on Monocyte Cell Surface CD14

Over a 4 hour culture period, the cell surface CD14 levels on ODN treated cells were not significantly different from the untreated cells at the same timepoint until t=4 at which time they had fallen to 28 ± 4% of baseline $p<0.001$ (Fig 4.1b).

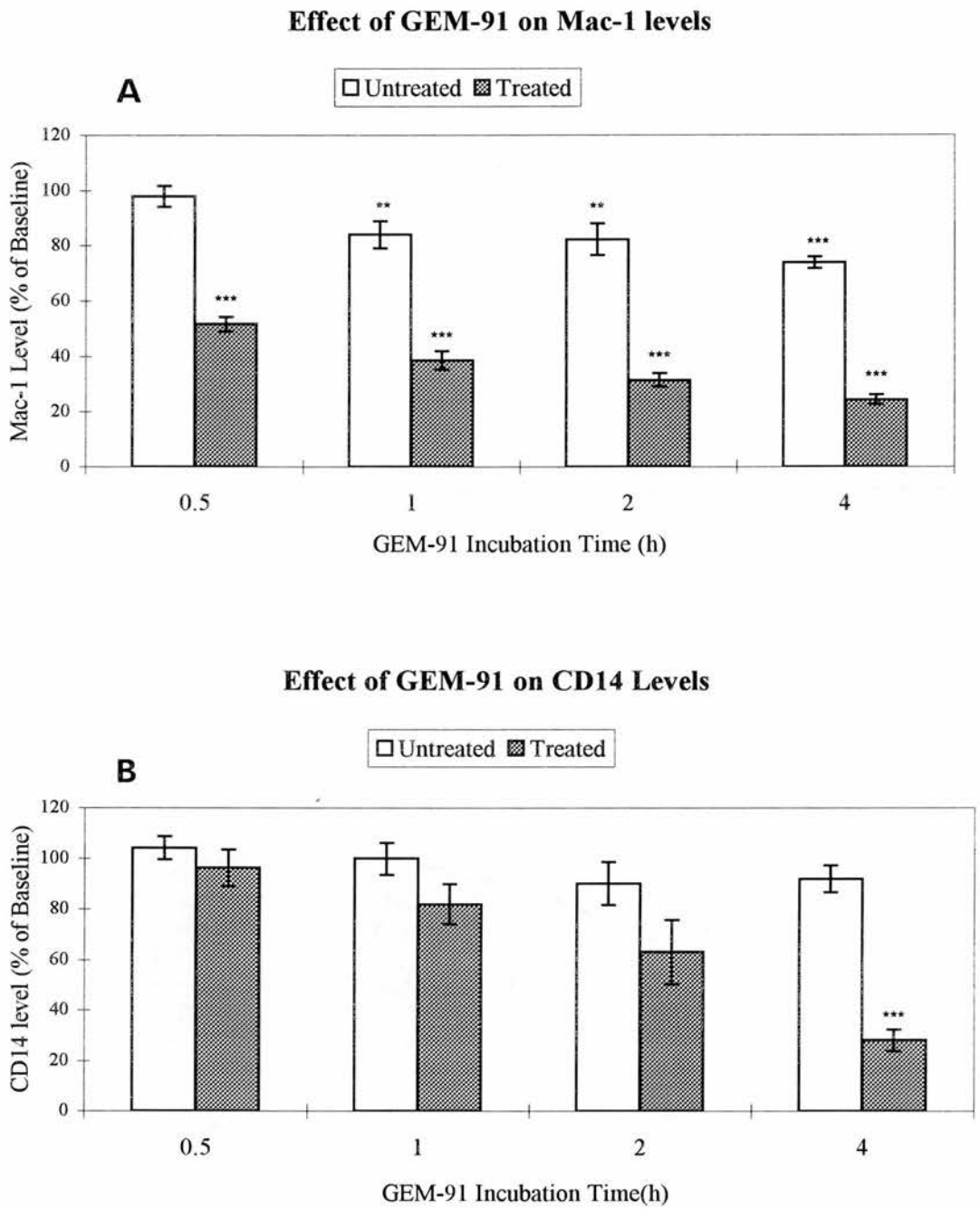


FIGURE 4.1 Effect of GEM-91 on Monocyte Mac-1 and CD14 Levels. PBLs were incubated with and without 5μM unlabelled GEM-1 for 4 hours. **A.** Monocyte cell surface Mac-1 levels decreased significantly over the 4 hour culture period, but in the presence of 5μM GEM-91 for 4 hours the Mac-1 levels decreased significantly further with respect to untreated cells. **B.** Cell surface CD14 levels did not significantly decrease until 4 hours into the culture period. In the presence of 5μM GEM-91 the cell surface CD14 levels decreased significantly with respect to the untreated cells.
** $p<0.01$, *** $p<0.001$, $n=6$.

4.3.4 Association Between Baseline Mac-1 Levels and GEM-91 Binding to PBL Cell Types

From figure 4.2 it can be seen that the cells having greatest cell surface Mac-1 levels (monocytes and neutrophils) bound the greatest amount of GEM-91 after 30 mins. In addition, it can be seen in figure 4.3 that cell surface Mac-1 levels on monocytes and neutrophils are higher than in lymphocytes.

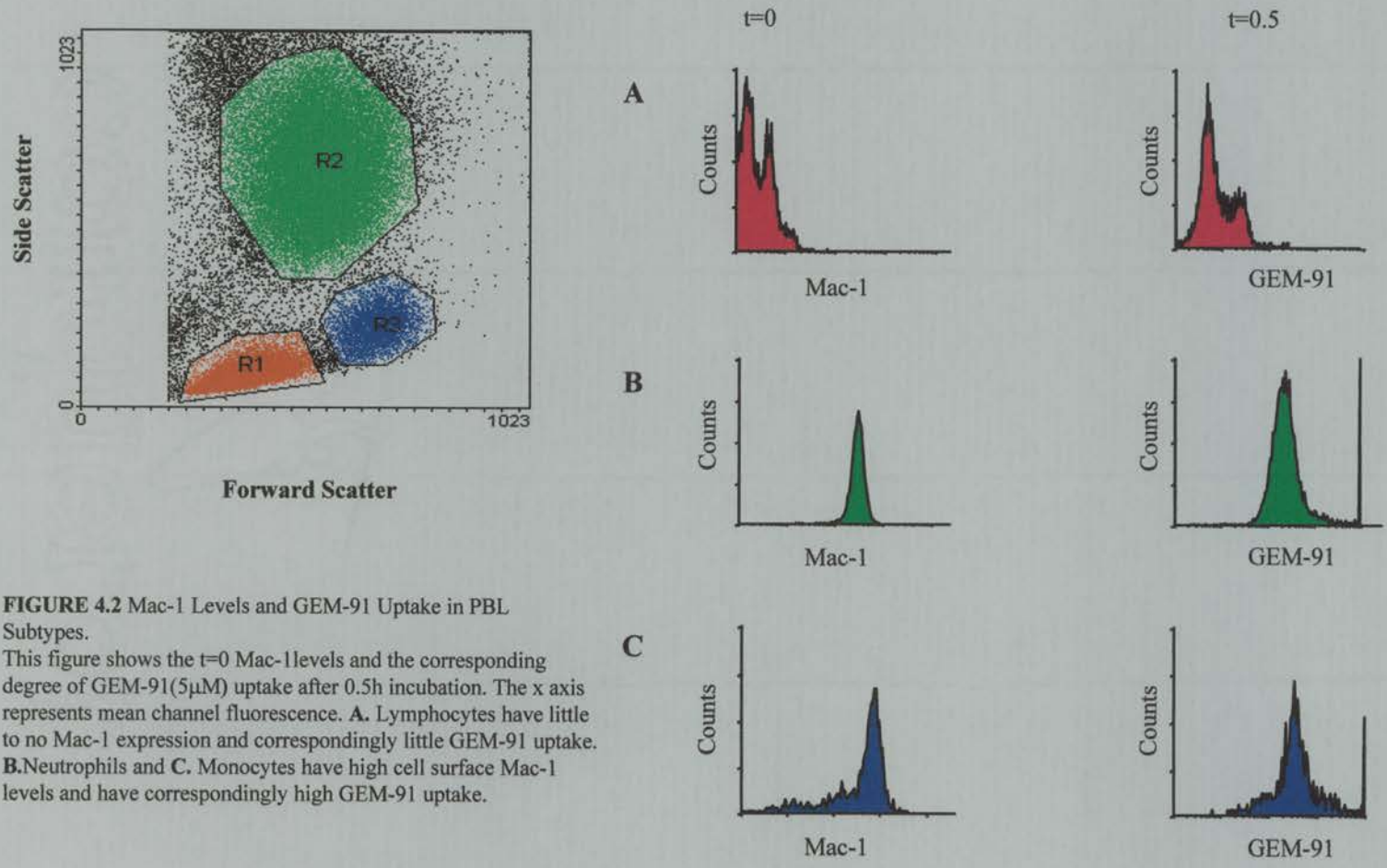


FIGURE 4.2 Mac-1 Levels and GEM-91 Uptake in PBL Subtypes.
This figure shows the t=0 Mac-1levels and the corresponding degree of GEM-91(5μM) uptake after 0.5h incubation. The x axis represents mean channel fluorescence. **A.** Lymphocytes have little to no Mac-1 expression and correspondingly little GEM-91 uptake. **B.**Neutrophils and **C.** Monocytes have high cell surface Mac-1 levels and have correspondingly high GEM-91 uptake.

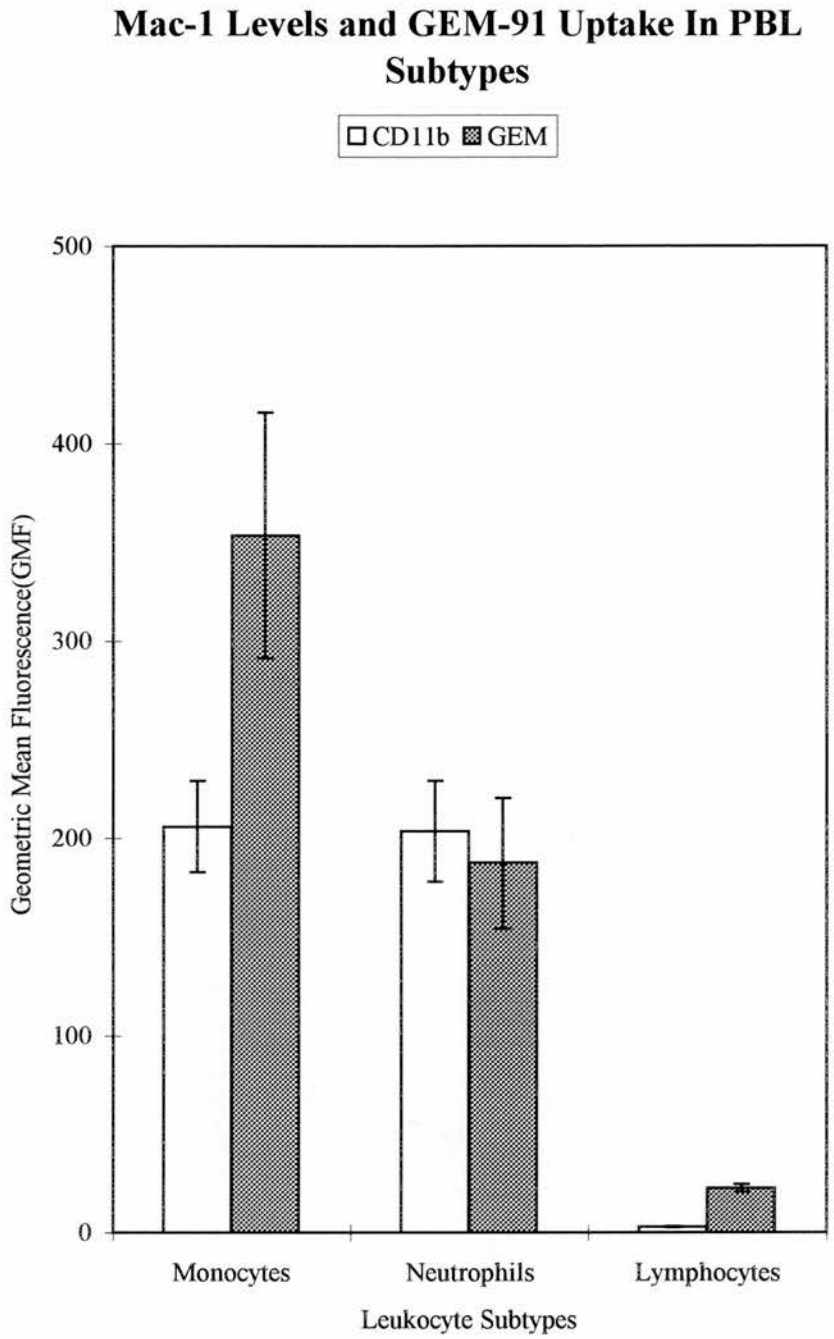


FIGURE 4.3 Mac-1 Levels on Peripheral Blood Leukocyte Subtypes and the Corresponding GEM-91 uptake. The Y axis represents the mean channel fluorescence of the cells. In the case of Mac-1 this is FL-2 Fluoresence, and in the case of GEM-91 it is FL-1 fluorescence. It can be seen that monocytes and neutrophils which have the greatest density of Mac-1 receptors also have greater GEM-91 uptake. Bars represent the mean of 6 separate experiments +/- sem.

4.4 Discussion

In the previous chapter it was shown that uptake of GEM-91 into PBLs is dose and concentration dependent over a four hour period and that the order of uptake efficiency among PBL subtypes after 4 hours is lymphocytes < neutrophils < monocytes. Many studies have been carried out that suggest that ODNs enter cells by receptor mediated endocytosis via a variety of uncharacterised cell surface proteins (Beltinger *et al.*, 1995, Kimura *et al.*, 1994). Recently, Benimetskya *et al.*, 1997 have demonstrated convincingly that the heparin binding integrin CD11b/CD18 (Mac-1) is a cell surface receptor for phosphodiester and phosphorothioate ODNs and is capable of mediating their internalization.

Using monoclonal antibodies to Mac-1 and CD14 directly conjugated with cy-5 and PE respectively, the cell surface levels of these receptors on monocytes were measured using flow cytometry with and without the addition of GEM-91. It has been shown that after 4 hours in culture monocyte cell surface levels of Mac-1 decreased to 73% of those detected at baseline. Conversely, culture conditions did not significantly decrease the cell surface CD14 receptor levels. In addition to the decrease in Mac-1 levels caused by culture alone, incubation with 5μM unlabelled GEM-91 caused further time-dependent decreases in the receptor levels such that after 30 minutes in culture, Mac-1 levels had significantly fallen and after 4 hours in culture Mac-1 levels had fallen to 24 ± 2% ($p<0.001$) of those at baseline. In contrast, in monocytes treated with GEM-91 CD14 levels did not fall significantly below levels in untreated cells until after 4 hours in culture. These results show that GEM-91 causes a rapid pronounced decrease in monocyte cell surface Mac-1 levels and an equally pronounced but slower decrease in cell surface CD14 levels. When FAM-labelled GEM-91 was incubated with PBMC in culture the highest levels of fluorescence were seen in the cells expressing Mac-1 namely monocytes and neutrophils. Lymphocytes had much lower associated GEM-91 (Figs 4.3 and 4.4). Despite the fact that neutrophils have a higher density of Mac-1 receptors than monocytes, it has been shown in the previous chapter and confirmed here that it is monocytes which internalize more ODN. This may be due to the fact that monocytes

have a greater density of cell surface receptors, in particular the CD14 receptor and these may be involved in ODN uptake. An alternative hypothesis might be that ODNs upregulate the expression of another receptor on monocytes, but not on neutrophils or lymphocytes, which results in an increased ODN binding. It has been suggested that ODN uptake is related to the cell cycle in that activated cells take up more ODN than non-active cells (Zhao *et al.*, 1996; Benimetskya *et al.*, 1997). Zhao *et al.*, 1995 have shown that ODN uptake was stage specific during B-cell differentiation. Because cell activation status may vary during cell maturation, it may be speculated that during monocyte maturation into macrophages, increased expression of cell surface receptors for ODNs may occur in addition to the increased phagocytotic ability of the cells contributing to an increased ODN uptake over and above that of neutrophils and lymphocytes. This activation could feasibly occur in the artificial conditions of cell culture.

The studies by Benimetskaya et. al., that demonstrate binding of ODNs to Mac-1 and other heparin binding proteins on the cell surface suggest that these receptors may be capable of internalizing ODNs. This observation in addition to the evidence from our study that the cells with highest levels of Mac-1 have the greatest associated ODN suggests that these receptors may be involved in uptake of ODN. Alternatively it is also possible that ODN binding to cell surface receptors may elicit an intracellular response initiating downregulation of the receptor or another response.

The present study shows that the extracellular presence of ODNs has an effect on monocyte cell surface receptors. Mac-1 levels quickly decrease after 30 minutes in the presence of ODN compared with CD14 levels which do not significantly decrease until 4 hours in culture with the ODN. This suggests that the ODN may be slowly changing the phenotype of the monocyte by downregulating the expression of CD14 and Mac-1. The effect of ODNs on the CD14 receptor would be relevant to studies concerning TF. The first step in the induction of TF by bacterial LPS is binding of the LPS molecule to the CD14 receptor. The simple binding of the ODN to the CD14 receptor, or downregulation of the receptor, would reduce the potential for LPS interaction with CD14 inhibiting the induction of TF. Conversely, interaction of the ODN with the CD14 receptor may actually induce TF expression (see chapter 6) by

setting in motion intracellular signalling pathways resulting in transcription of the TF gene. Although the current experiments suggest that, in agreement with Benimetskaya et al,. 1997, the Mac-1 and CD14 receptors are somehow involved in ODN uptake the results are far from conclusive. Studies examining ODN uptake in the presence of neutralizing antibodies to Mac-1 and CD14 or competitive ligands to these receptors would be needed to clarify actual ODN binding to these receptors.

CHAPTER 5

CHARACTERISATION OF LIPOPOLYSACCHARIDE INDUCTION OF TISSUE FACTOR IN WHOLE BLOOD

5.1 Introduction

Since monocytes are the only peripheral blood cell capable of expressing TF, there has been considerable interest in measuring the procoagulant activity of these cells in disease states. The results of these experiments have, however, been difficult to interpret mainly because of the diverse methodology employed. Functional approaches have utilized various methods for purifying monocyte populations, many of which artefactually activate the cells (Muller et al., 1985). Some studies have used clotting assays to quantitate TF activity (Østerud and Bjørklid, 1982), whilst others have used chromogenic methods to quantitate the amidolytic activity of the TF/VIIa complex (Carson et al., 1986). Interpretation is additionally complicated since some authors measure TF after cell disruption (Østerud and Bjørklid, 1982) whilst others evaluate intact cells (Surprenant and Zuckerman, 1989). Differences in the numeric expression of procoagulant activity adds to the problem. An alternative approach has been the use of flow cytometry (Luther et al., 1990; Carson et al., 1990), although it is often unclear whether TF antigen levels correlate with procoagulant activity.

5.1.2 Adherent Monocytes versus Monocytes in Suspension

Most of the information on the regulation of TF expression in monocytes stems from studies on adherent monocytes in cell cultures. LPS stimulation of adherent monocytes is apparently mediated through the protein kinase C system (Ternisiera et al., 1993) whereas observations by Østerud show that protein kinase C inhibitors fail to inhibit LPS induced TF activity in monocytes of whole blood. Furthermore, both the phorbol ester, phorbol myristate acetate (PMA) and TNF-α induce TF in adherent monocytes. In contrast, neither of these agents is capable of generating TF activity in monocytes of whole blood, although they enhance several fold LPS induced TF activity in these monocytes. Østerud has postulated that many of the agonists that apparently induce TF activity in adherent monocytes are merely enhancing the basal activity in already activated cells. In addition, monocytes allowed to adhere to culture plates for 24h do not express increased amounts of TF in response to LPS unless

lymphocytes are present in the cultures (Levy and Edgington 1980; Levy et al., 1981; Schwartz et al., 1981; Lyberg., 1983). Therefore, under certain circumstances, cellular co-operation is a prerequisite for TF expression in monocytes. Platelets and serum growth factors have also been shown to augment TF expression in LPS treated monocytes (Edwards and Perla 1984; Niemetz et al., 1974). These observations clearly suggest that the function and control of monocyte TF expression may not fully be appreciated by *in vitro* studies using isolated monocytes or monocytic cell lines.

5.1.3 Monocyte TF Induction In Whole Blood

This apparent difference in behaviour of monocytes in culture with those in suspension led Østerud to design an *ex vivo* whole blood model to investigate the induction of TF in monocytes in their own environmental milieu, in an attempt to decipher the intracellular co-operation involved (Østerud et al., 1982; Østerud et al., 1990). It has been shown that monocytes in whole blood appear to be activated entirely through the phospholipase A_2 (PLA_2) pathway to generate products like interleukins, TNFα and other cytokines as well as TF. In this system only two agonists, LPS and immune complexes, have been shown to induce TF activity. Using low dose LPS (2-5ng/ml) stimulated, anticoagulated whole blood as a test model, Østerud has characterized TF induction on monocytes. He has concluded that expression of TF activity is relatively constant in each individual tested, but varies between individuals. He reported a 50-fold difference in LPS induced activity between the highest and lowest responders (Østerud et al., 1994) but reported virtually no difference in TF antigen as measured by ELISA between the highest and lowest responders. This led him to speculate that the high TF activity induced in monocytes of the high responder was due to an increase in the catalytic activity of TF caused by an interaction between platelets and monocytes. In this whole blood system, he has also shown that PMA (60ng/ml) or physiological concentrations of TNF (5-10ng/ml) caused a 2 -3 fold enhancement of LPS-induced TF activity. In contrast, neither of these agonists alone was able to induce TF activity in this system.

It was postulated that the effects of these agonists were mediated by another cell type, possibly granulocytes or platelets.

5.1.4 Role Of Platelets In Expression of Monocyte TF Activity

In 1974, Niemetz suggested that platelets enhance the procoagulant activity of white blood cells (Niemetz et al., 1974). This was later confirmed in monocyte cell cultures where isolated platelets added to the cultures enhanced LPS induced TF activity (Lorenzet et al., 1985; Pinder et al., 1985). Furthermore, 12-Hydroxy-eicosatetraenoic acid (HETE), a platelet product, was claimed to enhance LPS-induced TF activity in adherent monocytes (Lorenzet et al., 1985). Østerud further confirmed this platelet effect in his whole blood model. He removed platelets from the white blood cells and then cross combined the white cells with either platelet rich plasma (PRP) or platelet poor plasma (PPP). He subsequently stimulated the system with LPS and observed a significant platelet enhancing effect on LPS induced TF activity (Østerud et al., 1990). Østerud then went on to demonstrate that the variation in LPS induced TF activity between individuals was partly attributable to platelets. Thus recombining platelet poor cells from high responders with platelets from low responders lead to a marked reduction (60-70%) in LPS induced TF activity. Accordingly, a significant rise in TF activity was observed when platelets from high responders were recombined with cells from low responders. A mandatory role for granulocytes in providing the platelet enhancement of LPS-induced TF activity in monocytes has also been demonstrated by this group (Halvorsen et al., 1993). In addition, exogenously added platelet activating factor (PAF) enhanced LPS-induced TF activity in monocytes provided that both granulocytes and platelets were present (Østerud et al., 1992). Furthermore, a PAF antagonist was shown to suppress LPS induced TF activity in monocytes.

It is, therefore, apparent that in order to fully investigate TF induction by LPS in monocytes, a whole blood model should be used to facilitate physiological cell interactions. We have characterized TF induction by LPS in whole blood, examined

the reproducibility of this induction both within subjects and between subjects and have looked at the ODN association with PBLs in a whole blood environment.

5.2 Materials and Methods

5.2.1 Blood Collection and Processing

Whole blood was collected from 6 healthy volunteers by clean venepuncture with a 21 gauge needle into sterile vacutainers (Sarsdtet) containing 3.2% trisodium citrate anticoagulant.

5.2.2 Dose-Response and Timecourse of TF Induction on Monocytes by LPS

Blood aliquots (1ml) were placed in sterile 15ml polypropylene tubes (Falcon) and treated with LPS from *E. coli* O111:B4 (Sigma) (reconstituted in sterile distilled water) to produce final concentrations of 0, 10pg/ml, 1ng/ml, 100ng/ml, 10μg/ml, 100μg/ml. *E. Coli* stock solutions were made up such that 20μl volumes were added to each sample to produce the desired final concentration. The samples were incubated at 37°C with 95% air, 5% CO_2. Aliquots (100μl) were removed at 2h, 4h, 6h, 24h, and were immediately indirectly immunostained for TF as described in chapter 2. The primary monoclonal antibody cocktail to TF was kindly donated by Prof. T.S.Edgington, Scripps Research Centre, La Jolla, CA.

5.2.3 Flow Cytometry

Fixed, stained, cells were analysed by flow cytometry as described in chapter 2. Briefly, 50000 cells were collected and monocytes were identified by virtue of their forward and side scatter characteristics and were periodically checked to be 95% CD14 positive. Isotype control stained cells were used to set the positive delineator at 3% positive cells. Subsequently, cells falling within this marker were deemed to be positive for TF. In addition, the geometric mean fluorescence of the TF stained cells was recorded along with the geometric mean fluorescence of an isotype control at each dose and timepoint.

5.2.4 Person Specific TF Induction

6 normal healthy males were recruited to take part in a repeated study to look at monocyte TF induction in individuals over a 3 month period. Blood was collected from these subjects on 4 or 5 different occasions over a three month period. The blood was stimulated with either 100ng/ml LPS or 10μg/ml for 2 hours and monocyte TF levels measured as above by flow cytometry.

5.2.5 Association of GEM-91 with PBLs in Whole Blood vs Isolated Cells

As we were now using a whole blood model to look at TF induction in monocytes with a view to it's inhibition with ASODNs we investigated the association of GEM-91 to monocytes in whole blood compared with that in isolated peripheral blood leukocytes. The ODN overall association was not differentiated into an intracellular and an extracellular component as was the case in chapter 3, due to the expense of the ASODN tested. However, it was assumed that cell surface bound ODN would be internalised to the same extent as in chapter 3. Blood was collected and leukocytes isolated as described previously. Aliquots either of whole blood (500μl) or peripheral blood leukocyte suspension in QBSF media (500μl of $1x10^7$ cells) were incubated for 4 hours with either 0, 0.1, 1.0 or 5 μM final concentration of GEM-91. The whole blood aliquots were then treated with red cell lysis solution as before and washed, as were the isolated cells twice in PBS. The cells were then analysed by flow cytometry as described above (50000 cells collected and geometric mean channel fluorescence measured on the FL1 channel).

5.3 Results

5.3.1 Dose Response and Timecourse of LPS Induction in Monocytes

After a 2h incubation with increasing doses of LPS, there was a dose dependent increase in TF expression on monocytes in whole blood (Figs. 5.1 and 5.2). At baseline there was a mean of 1.03 ± 0.26 % positive cells (geometric mean fluorescence GMF 0.02 ± 0.02). A 2h incubation with sterile distilled water produced 3.27 ± 0.68 % positive cells (GMF 0.25 ± 0.13). On the addition of increasing concentrations of LPS (10pg/ml, 1ng/ml, 100ng/ml, 10μg/ml and 100μg/ml) the TF expression increased to 4.61 ± 1.91 % positive cells (GMF 0.61 ± 0.34), 23.12 ± 4.13 % positive cells (GMF 2.46 ± 0.91), 53.93 ± 8 % positive cells (GMF 8.96 ± 2.04), 66.04 ± 9.53 % positive cells (GMF 17.14 ± 4.77). At this timepoint the isotype control antibody staining was consistently low. At 4h, 6h, 24h incubation times, the cell morphology gradually changed, possibly due to monocyte differentiation into macrophages, and it became increasingly difficult to distinguish monocytes from the other peripheral blood leukocytes. The isotype control antibody also began to stick to the cells and stain positive. This decline in monocyte quality over longer incubation times suggested that a 2h incubation be used for all subsequent induction experiments as this seemed to be the limit to our experimental system.

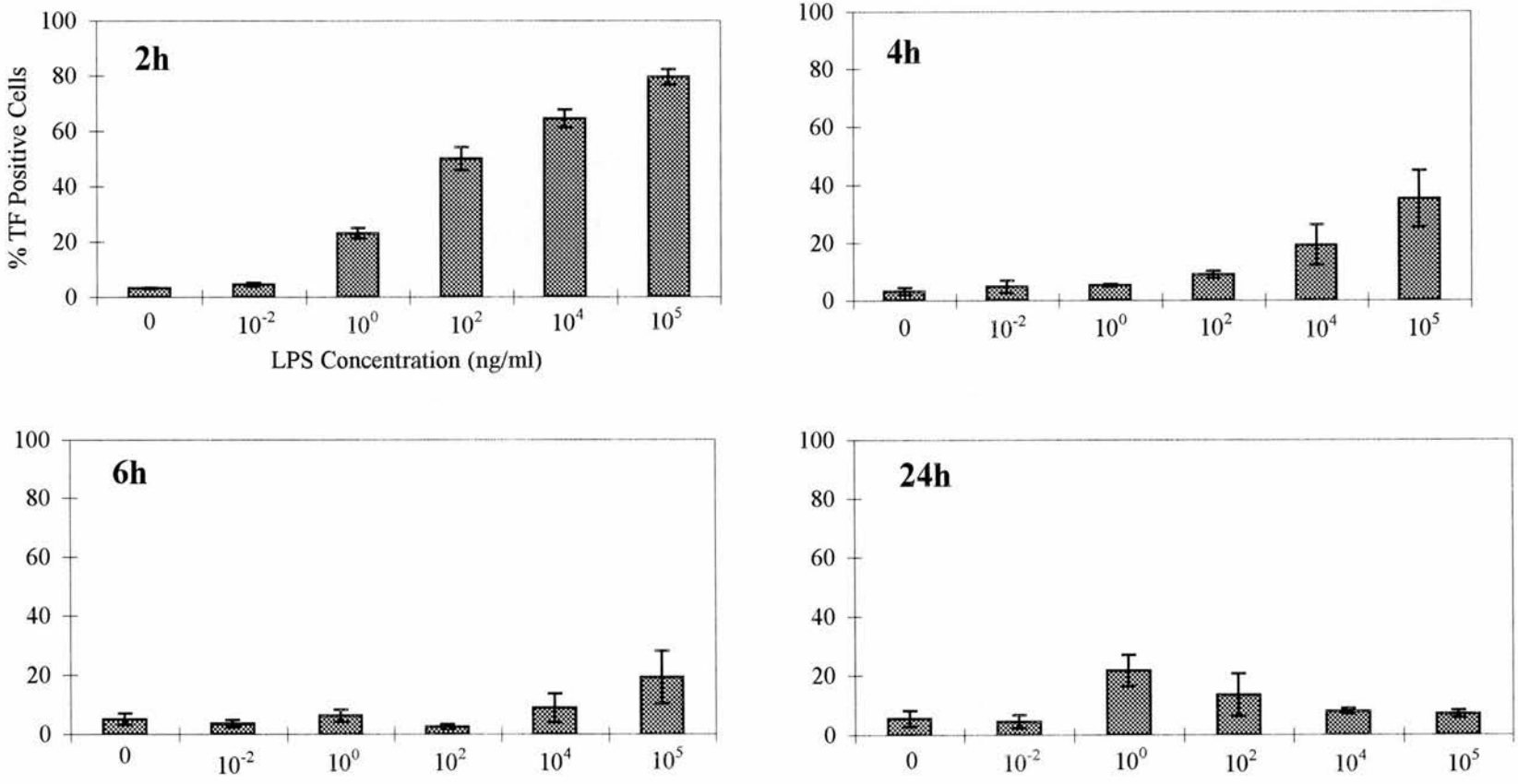


FIGURE 5.1 Dose-response and Timecourse of MonocyteTF Induction by LPS (%positive cells). Increasing concentrations of LPS were added to whole blood aliquots and incubated for up to 24h. Monocytes were then immunostained for TF and analysed by flow cytometry. Background staining was determined using an isotype control antibody and from this the percentage positive monocytes was determined. It can be seen that a 2h incubation time produced an optimal dose-response relationship. Bars are the mean of 8 experiments +/- sem.

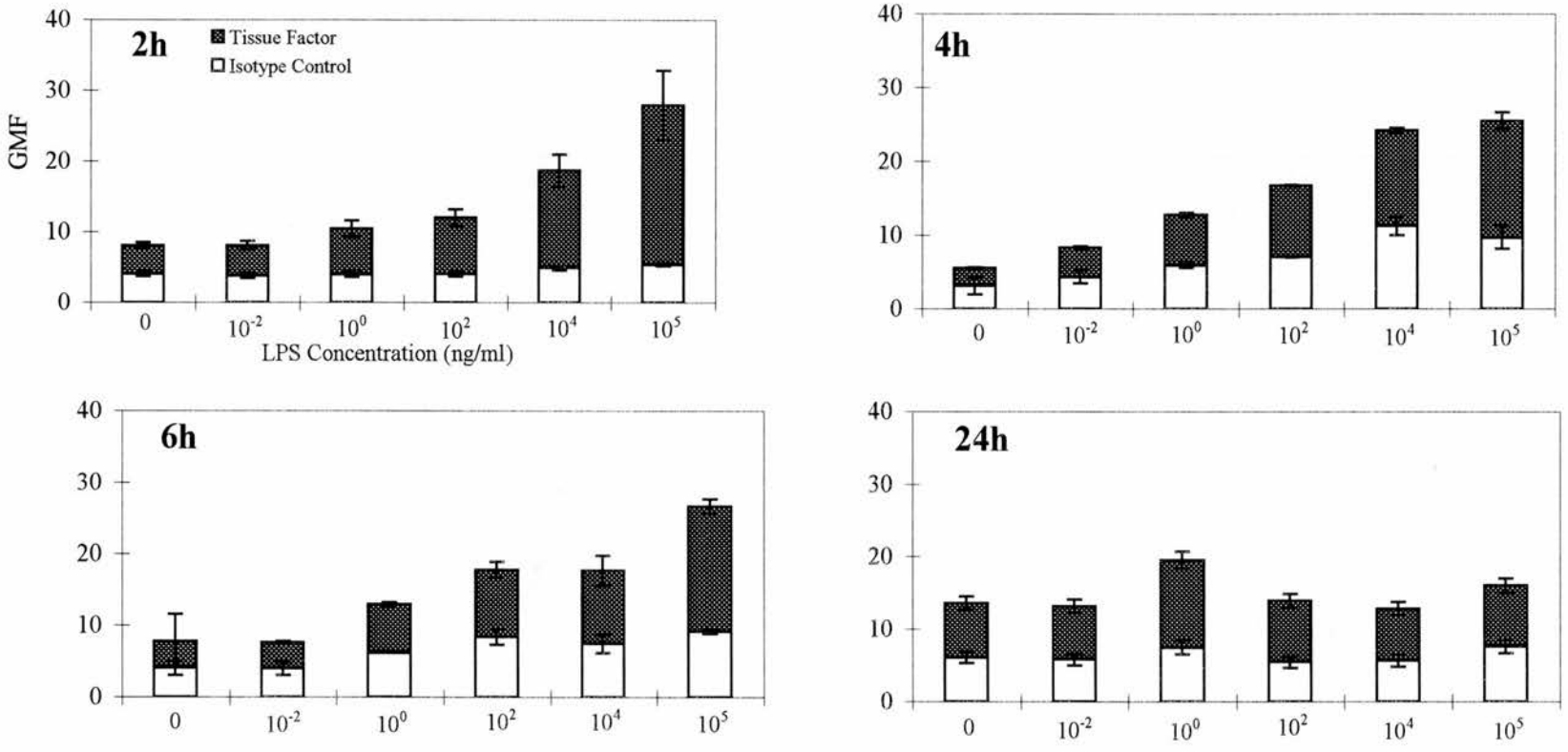


FIGURE 5.2 Dose response and Timecourse of MonocyteTF Induction by LPS (Geometric Mean Channel Fluorescence). Increasing concentrations of LPS were added to whole blood aliquots and incubated for up to 24h. Monocytes were then indirectly immunostained with a TF mAb or isotype control antibody and analysed by flow cytometry. The geometric mean channel fluorescence of the monocytes was recorded. It can be seen that after 4 hours incubation with LPS the isotype control antibody binds increasingly to monocytes. For this reason a 2 hour incubation with LPS was the limit to our experimental design. Bars are the mean of 8 separate experiments +/- sem.

5.3.2 TF Induction in Individual Subjects

Six healthy volunteers were recruited to participate in a 3 month study to test the reproducibility of the whole blood assay both within the same individual and between individuals. Blood was taken from each individual on up to 5 occasions over a 3 month period and was treated with two concentrations of LPS for 2 hours. The coefficient of variation was calculated for tests done on the same person over the three month period (Table 5.1). In addition, mean values were calculated for each dose on each individual and were compared with each other (Fig 5.3). All results are expressed as percentage **TF positive monocytes**.

It was apparent that the at the higher dose of 10μg/ml LPS there was a reproducible intra-person induction of TF on whole blood monocytes. In 5 of the six subjects tested repeatedly over the course of 3 months the cv < 10%, and in the individual with large variability (KW), the isotype control stained samples were also high, suggesting that this individual had particularly reactive monocytes. However, at the lower dose of 100ng/ml LPS there was much more variation in the monocyte TF measured on the same individual on different occasions, the cv being frequently greater than 20%. Similarly, there was great variation in the TF induced by incubation with distilled water alone in blood from the same individual tested on different days the cv ranging from 18-80%.

Subject	Isotype Control	0 LPS	100ng/ml LPS	10μg/ml LPS
DS	1.57 (5)	34.5 (5)	11.1 (4)	8.88 (5)
SB	2.34 (5)	18.4 (5)	2.88 (5)	3.7 (5)
KW	21.5 (5)	77.1 (5)	29.3 (5)	16.2 (5)
RW	2.8 (5)	80.9 (5)	31.6 (4)	6.92 (5)
DF	2.51 (5)	51.0 (5)	16.6 (5)	9.41 (5)
RM	2.6 (4)	28.6 (4)	16.1 (3)	7.63 (4)

TABLE 5.1 Coefficients of Variation in Each Individual When Treated with 2 Concentrations of LPS (% positive cells). Numbers in brackets denote number of times tested over 3 month period. (cv=%).

With respect to inter-person variation, at the 100ng/ml LPS dose, there was a maximum of a 1.75 fold difference in the lowest (36.3 ± 2.0% positive monocytes, n=4) and the highest (63.7 ± 5.9% positive monocytes, n=3) responders. At the higher dose of 10μg/ml LPS there was very little inter-person variation the lowest responder having 61 ± 2.4% positive monocytes, n=5, and the highest responder having 78.11 ± 5.6% positive moncytes, n=5.

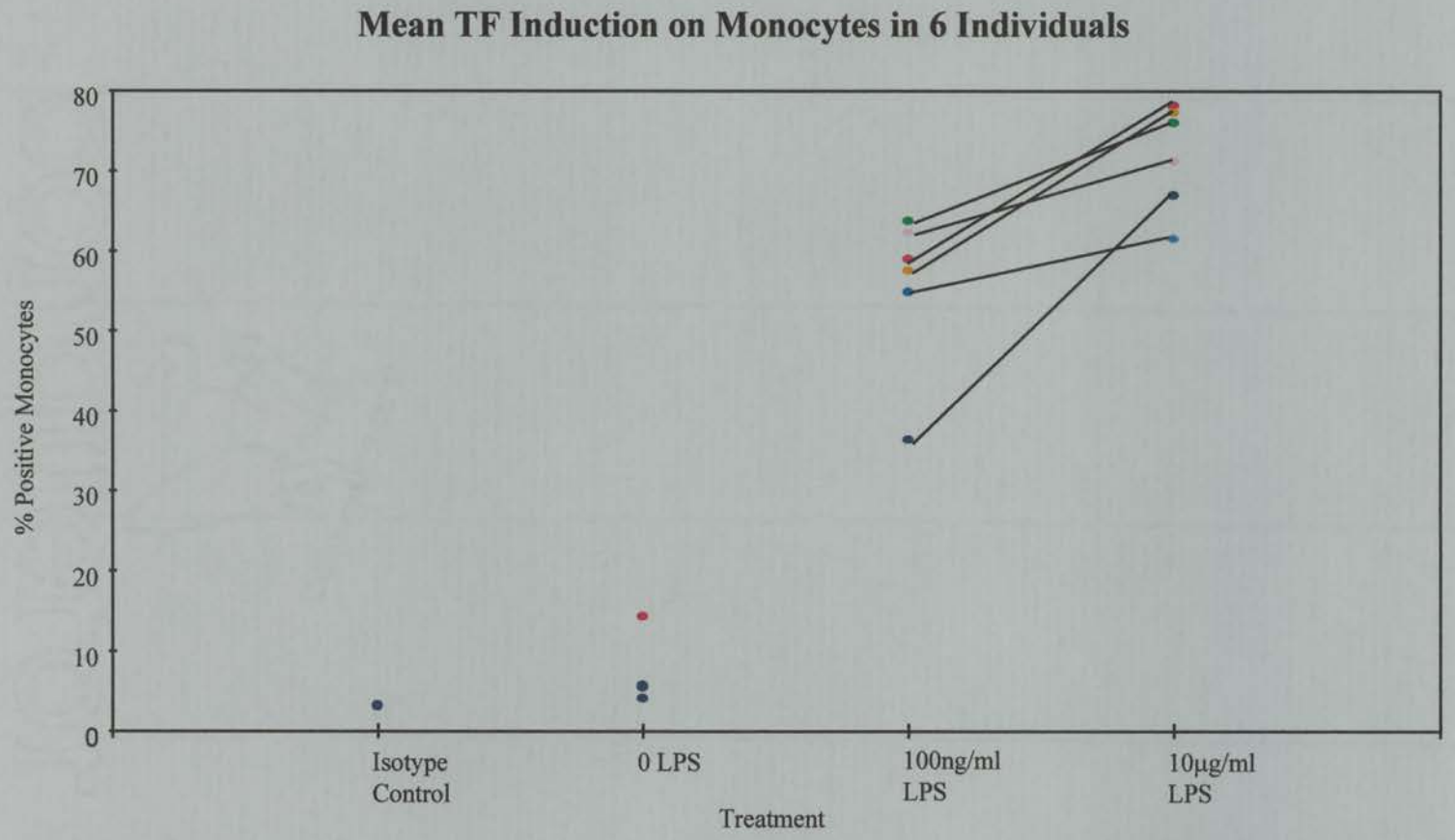


FIGURE 5.3 % TF Positive Monocytes in 6 Individual Subjects. 6 healthy volunteers were recruited and their blood treated with 2 concentrations of LPS on 4 -5 different occasions over a 3 month period. The mean of these repeated tests is shown, along with lines linking tests from the same subject. Standard error bars are not shown for ease of viewing.
The control tests (treated with 10µg/ml LPS) represent n=5, however, this is not apparent due to co-incidence of data.

5.3.3 Association of GEM-91 with PBLs in Whole Blood vs Isolated Leukocytes

5.3.3.1 Total Cells

In general, association of GEM-91 with PBLs in whole blood was overall less than in isolated cells for each cell type examined (Figs.5.4a-d). At 0.1μM GEM-91 the total fluorescence associated with the cells (GMF) was 2.52 ± 0.024 in whole blood vs 31.3 ± 11.9 in isolated cells. At the higher dose of 1.0μM the total fluorescence associated with the cells was 4.04 ± 0.2 for whole blood vs 56.9 ± 16 for isolated cells. Finally, at 5μM GEM-91 the total cellular fluorescence was 15.86 ± 2.4 for whole blood vs 60.4 ± 19 for the isolated cells. However, the pattern of ODN association in the different cell types was the same in whole blood as in isolated cells, with monocytes having the greatest association of ODN, followed by neutrophils and finally lymphocytes having the least.

5.3.3.2 Lymphocytes

Lymphocytes took up very little ODN either in whole blood or in isolated blood cells. At an ODN dose of 0.1μM there was 7 fold less associated with lymphocytes in the whole blood model than in isolated cells (GMF 1.4 ± 0.04 vs 7.1 ± 1.9). At the 1μM dose GMF was 2.4 ± 0.4 in whole blood vs 14.5 ± 2.9 in isolated cells and at the 5μM dose, GMF was 6.8 ± 1.4 in whole bloodvs 17.9 ± 2.8 in isolated cells.

5.3.3.3 Monocytes

The monocyte population had the greatest associated ODN both in the whole blood setting and in the isolated cells preparation. At GEM-91 concentrations of 0.1 and 1.0 μM association with monocytes was 10 fold less in whole blood than in isolated cells after a 4 hour incubation (GMF 12.3 ± 0.2 vs 131.3 ± 36.7 and 41.9 ± 1.3 vs 407.9 ± 68.8 respectively). At the 5 μM dose of GEM-91 association with

monocytes in whole blood was 3.5 fold less than in isolated cells (GMF 140.4 ± 11.8 vs 502.1 ± 86.2).

5.3.3.4 Neutrophils

Oligonucleotide association with neutrophils was approximately 20 fold less in whole blood than in isolated cells (GMF 3.2 ± 0.2 vs 63.1 ± 19) at a GEM-91 concentration of 0.1μM. At a 1μM dose, neutrophil associated ODN was 40 times less in whole blood than in isolated cells (GMF 6.4 ± 1.2 vs 251 ± 90.5). At the 5 μM dose there was a 12 fold greater ODN association with neutrophils in isolated cells compared with neutrophils in whole blood (GMF 27.2 ± 3.8 vs 324.7 ± 27.2).

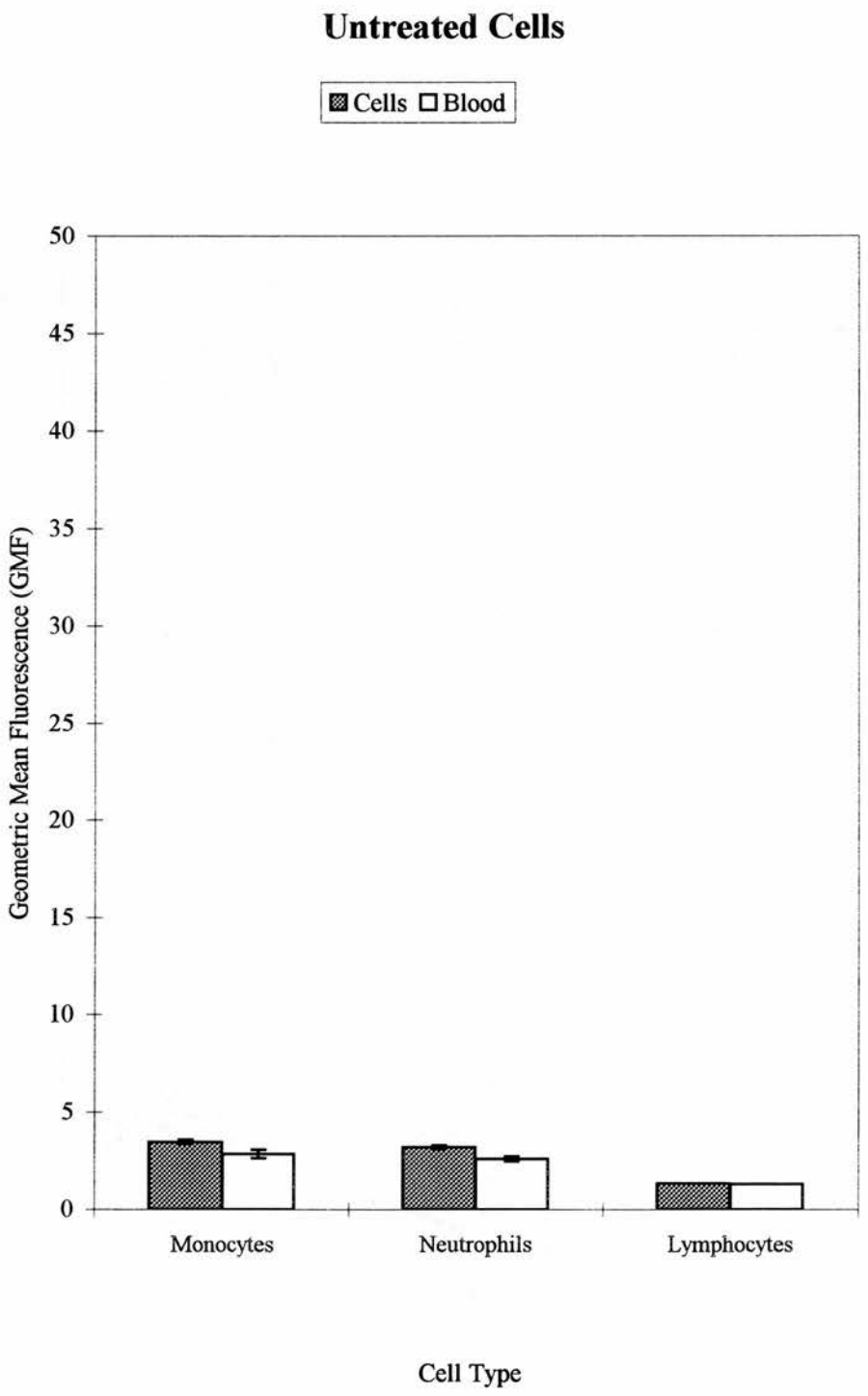


FIGURE 5.4a Geometric Mean Fluorescence of untreated isolated cultured PBLs and PBLs in whole blood.
All the cell types tested had a low background fluorescence. Bars represent the mean of 4 separate experiments +/- sem.

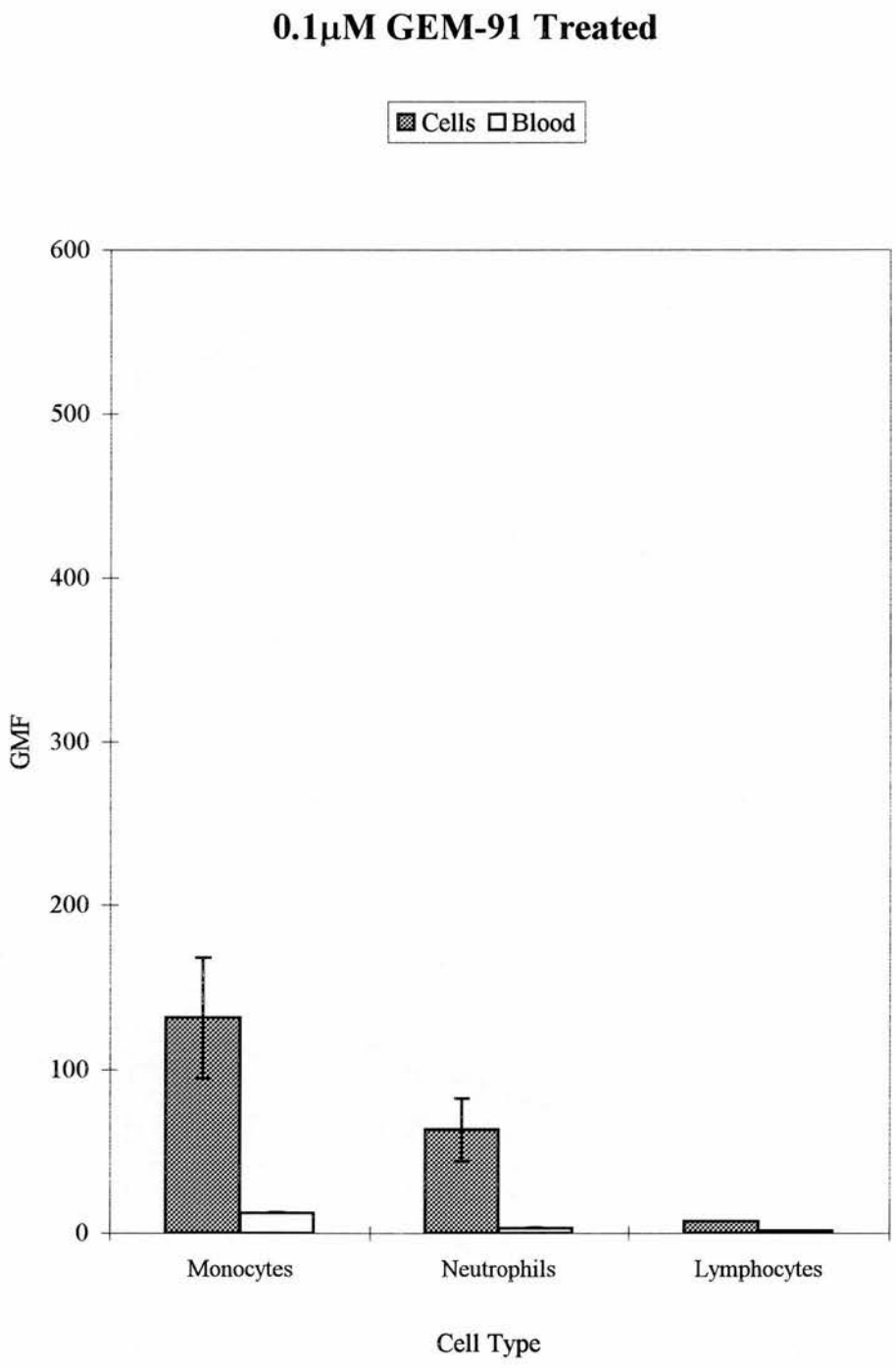


FIGURE 5.4b Geometric Mean Fluorescence (GMF) of 0.1μM FAM-labelled GEM-91 treated isolated PBLs or PBLs in whole blood. It can be seen that PBLs isolated from GEM-91 treated whole blood have less associated ODN than GEM-91 treated freshly isolated PBLs in culture. In both cases monocytes have the greatest associated ODN. Bars represent the mean of 4 separate experiments +/- sem.

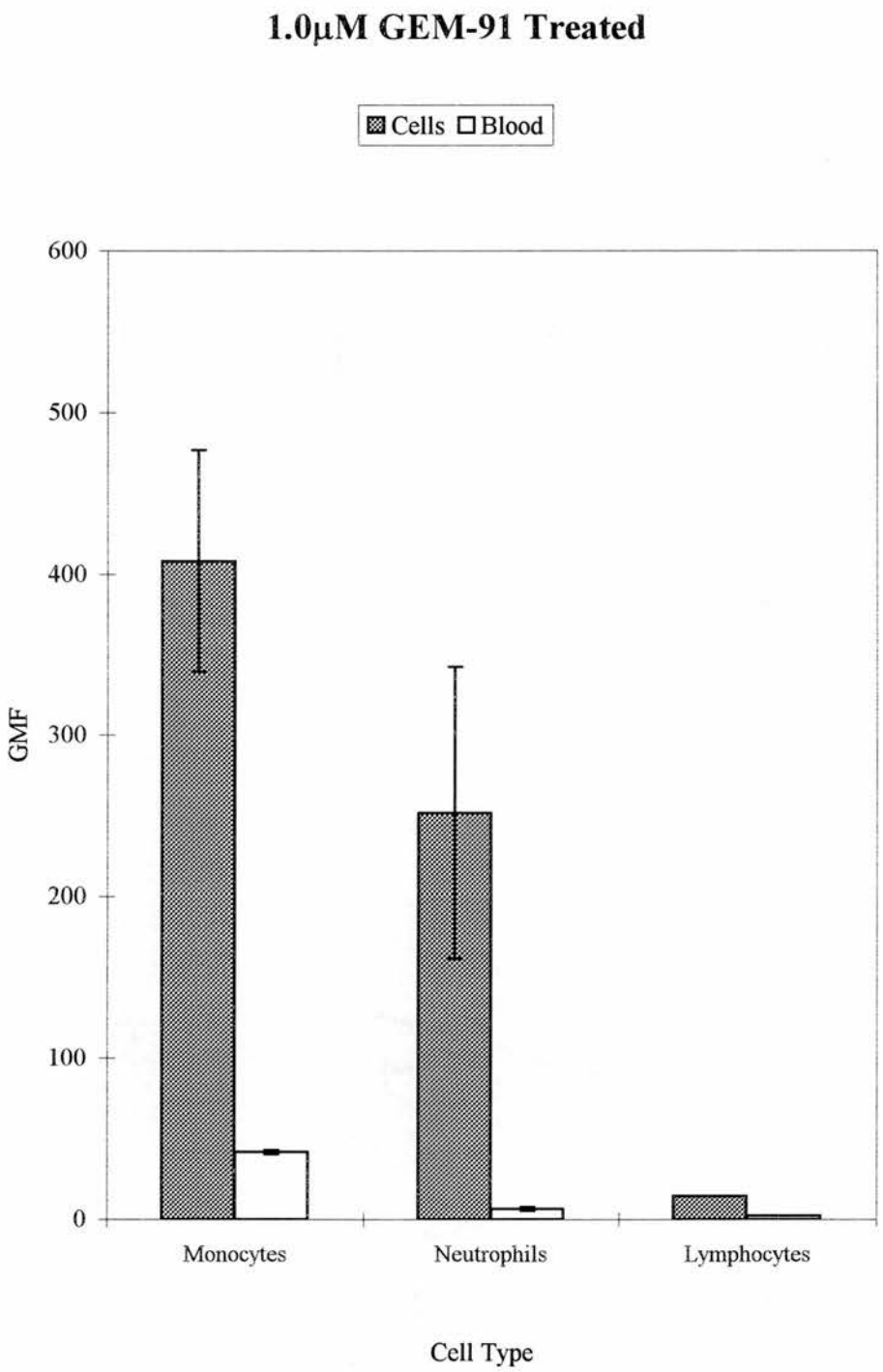


FIGURE 5.4c Geometric Mean Fluorescence (GMF) of 1.0μM FAM-labelled GEM-91 treated isolated PBLs or PBLs in whole blood. PBLs extracted from GEM-91 treated whole blood have less associated ODN than GEM-91 treated freshly isolated PBLs in culture. In both cases monocytes have the greatest associated ODN. Bars represent the mean of 4 separate experiments +/- sem.

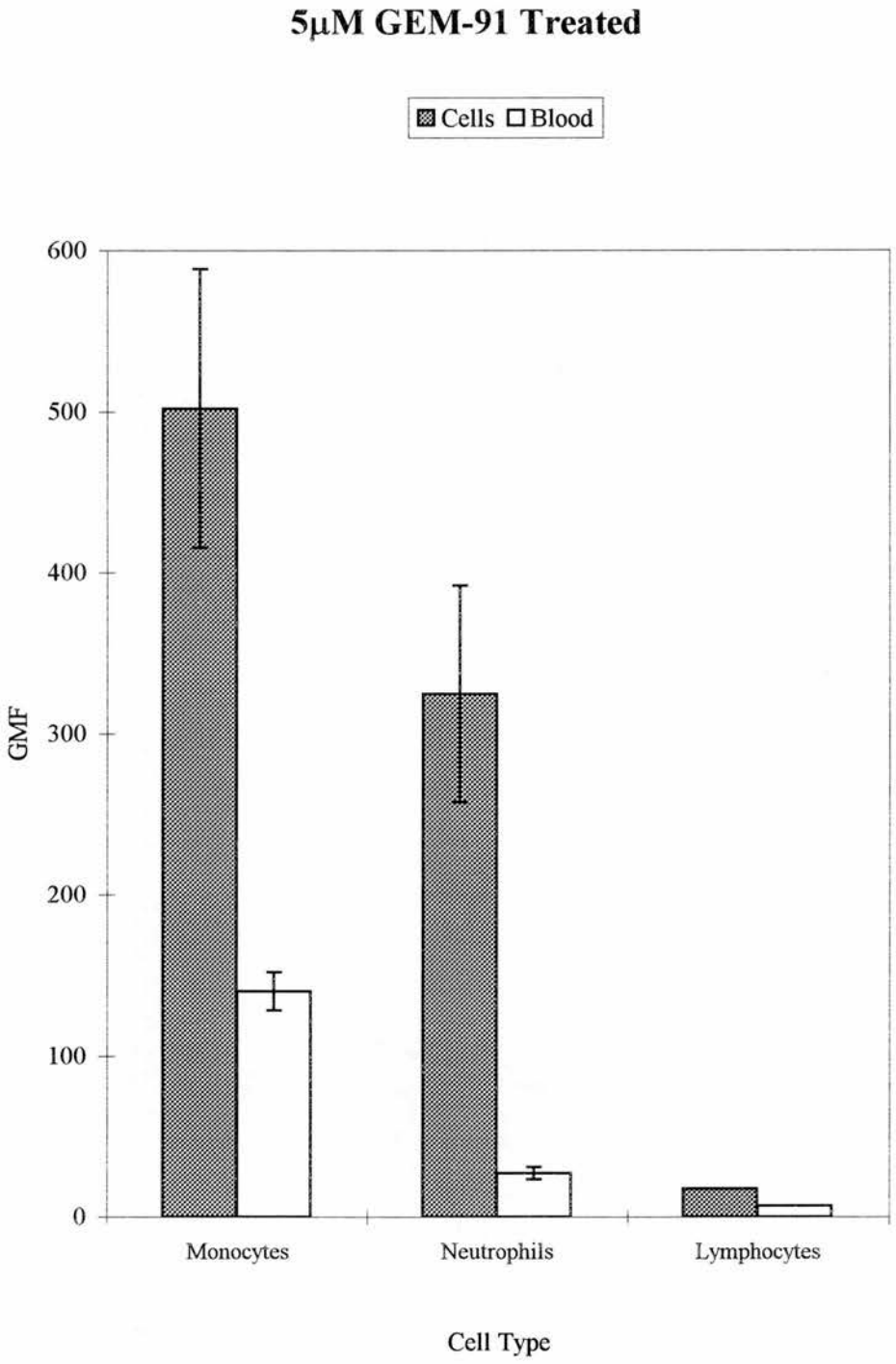


FIGURE 5.4d Geometric Mean Fluorescence (GMF) of 5.0μM FAM-labelled GEM-91 treated isolated PBLs or PBLs in whole blood. PBLs extracted from GEM-91 treated whole blood have less associated ODN than GEM-91 treated freshly isolated PBLs in culture. Again, in both cases monocytes have the greatest associated ODN. Bars represent the mean of 4 separate experiments +/- sem.

5.4 Discussion

Monocytes are the only peripheral blood mononuclear cell capable of expressing TF when stimulated and as such represent an important source of systemic procoagulant activity. Most of the information available on the regulation of TF expression stems from studies on monocytes in culture (Muller et al., 1985; Luther et al., 1990). Østerud is of the opinion that cultured, adherent monocytes do not accurately represent the pathophysiological reactions and signal transductions involved in the expression of TF in circulating blood *in vivo*, and for this reason, he believes that the results of these studies provide information on a particularly artificial situation. In an attempt to improve understanding of monocyte behaviour in their own environmental milieu, Østerud et al 1982, designed an *ex vivo* whole blood model to investigate monocyte TF expression. In the decade that followed, Østerud built up a picture of the behaviour of monocytes in whole blood when stimulated to express TF. He concluded that LPS was capable of inducing TF activity in monocytes in whole blood and that although this induction of was relatively constant in each individual, there was up to a 50-fold difference in LPS-induced activity between the highest and lowest responders at concentrations of LPS between 2-5ng/ml (Østerud et al., 1994). However, he also documented that there was virtually no difference between TF antigen expression between the highest and lowest responders when measured by Enzyme Linked Immunosorbent Assay (ELISA).

More recently Amirkhosravi et al., 1996 have developed a 2-colour whole blood cytometric technique to measure cell surface TF antigen in monocytes in whole blood using a pre- FITC-conjugated monoclonal antibody to TF in conjunction with a fluorescently labelled CD14 monoclonal antibody. Using small volumes of citrated anticoagulated blood stimulated with 10μg/ml *E. coli* O111:B4 LPS they have shown that monocyte TF antigen levels in normal subjects are low, 1.1 ± 0.95% making small increases easy to detect. Percentage TF antigen positive monocytes following LPS stimulation at 10μg/ml for 1 hour was 34.6% ± 11.2%. Furthermore, they observed that LPS-stimulated, TF antigen positive monocytes varied between subjects

(21-68%) a 3 fold difference. This was in contrast to Østerud's observation that although TF activity varied by as much as 50-fold between the highest and lowest responders, there was no measurable difference in TF antigen (Østerud et al., 1994). This discrepancy may be due to the fact that Østerud used a much lower LPS concentration than Amirkosravi et al., (2ng/ml vs 10μg/ml), used heparin as anticoagulant rather than citrate and measured clotting activity rather than antigen. It may also be due to the fact that flow cytometry is a more sensitive method of detecting cell surface antigens enabling smaller fluctuations in TF antigen to be detected than is possible with ELISA. Amirkosravi et al., also demonstrated that stimulated monocyte TF expression within individuals was relatively constant (cv= 5.4%) a fact also noted by Østerud (1995a and 1995b).

In addition, Amirkosravi et al., has also shown that there is a strong positive correlation between monocyte TF as measured in whole blood by flow cytometry and procoagulant activity as measured by whole blood recalcification time using a sonoclot analyser, suggesting that the expressed TF antigen is functionally active.

In the light of these conflicting reports, we have sought to characterize the time and dose dependent induction of TF in a whole blood model using flow cytometry, which appears to be the most sensitive method of detecting monocyte TF antigen expression.

Therefore, the purpose of this study was 3 fold. Firstly, it was required to establish, in my hands, the timecourse and dose-response relationship of LPS-induced TF expression in monocytes in a whole blood setting. This was necessary to determine an appropriate LPS dose and incubation time to provide optimal TF induction, whilst maintaining sufficient monocyte morphology and integrity to facilitate flow cytometric analysis. Secondly, it was necessary to assess the reproducibility of this whole blood model of TF induction by LPS, both between different individuals, and in the same individual tested on different occasions. We have developed an *ex vivo* whole blood assay to investigate LPS induction of TF, designed to facilitate flow cytometric measurement of TF antigen. The third aim of this study was to examine the association of GEM-91 with monocytes when incubated with whole blood in our *ex vivo* system.

In agreement with Amirkhosravi et al., 1997, a low TF antigen level at baseline was observed (1.03 ± 0.26% positive monocytes GMF 4.04 ± 0.45) making it easy to detect the dose dependent increase in the percentage positive over the range of LPS concentrations tested (0, 10pg/ml, 1ng/ml, 100ng/ml, 10μg/ml and 100μg/ml) after a 2 hour incubation (3.27 ± 0.68%; 4.61 ± 1.91%; 23.12 ± 4.13%; 50.1 ± 3.19%; 64.41 ± 2.78% and 79.35 ± 3.81% positive monocytes) (Fig. 5.1a). However, incubation times longer than 2h resulted in a breakdown of the dose-response relationship (Fig 5.1b-d). As can be seen in figure 5.2b-d the samples immunostained with isotype control antibody (control not designed to bind to TF) stained positive. As incubation time with LPS increased, especially at the higher doses of LPS, monocyte morphology was seen to deteriorate, making it difficult to distinguish them from lymphocytes on the FACS plot. In addition, the monocytes became increasingly 'sticky', binding control primary antibody and fluorescently labelled secondary antibody at random. Consequently, it was decided that a 2 hour incubation with LPS was the limit to the experimental set-up, and this time was chosen for subsequent stimulation experiments. Similarly, Amirkhosravi, et al chose a 1 hour incubation with LPS in their set-up. They observed a near maximal TF induction at this timepoint, and a corresponding fast clotting time.

Six healthy male volunteers were recruited to take part in a 3 month study to determine the reproducibility of the whole blood flow cytometric assay for monocyte TF. Blood samples from each of the six individuals were taken between 3 and 5 times over the 3 month period and were incubated in the absence and presence of LPS (100ng/ml and 10μg/ml) for 2 hours. Monocyte TF antigen was then determined by flow cytometry. Table 1 shows that the reproducibility of the assay within individuals at 10μg/ml LPS was much better than when the subjects were treated with the lower dose of 100ng/ml LPS. The coefficients of variation (cv) were below 10% in all but one case at the 10μg/ml dose whereas the cvs were often over 10% at the lower dose of 100ng/ml. In the individual with the higher cv at 10μg/ml, it can be seen that there was a corresponding variation in the isotype control stained cells (cv=21.5%). This suggests that this individual may have monocytes particularly susceptible to activation

and thus background staining during these incubation conditions. Østerud et al., 1992 have also reported a relatively constant induction of TF activity within individuals. However, his experiments were carried out at doses of LPS much lower (between 2-5ng/ml) than our lowest dose of 100ng/ml. The lack of reproducibility at 100ng/ml seen in the current study may be due to the fact that citrate was used as an anticoagulant compared with heparin used by Østerud. However, Amirkhosravi et al., 1996 who have reported a 5% coefficient of variation within individuals at a dose of 10μg/ml have reported that they observed no difference between the anticoagulants heparin and citrate. Østerud measured TF activity in lysed cells and not the TF antigen on intact monocytes. In addition, the sensitivity of the flow cytometric technique used in the current study, may make it easier to detect intra-person fluctuations in TF antigen

With respect to inter-person variation, at the 100ng/ml LPS dose, there was a maximum of a 1.75 fold difference in the lowest (36.3 ± 2.0% positive monocytes, n=4) and the highest (63.7 ± 5.9% positive monocytes, n=3) responders. At the higher dose of 10μg/ml LPS there was very little intra-person variation, the lowest responder having 61 ± 2.4% positive monocytes, n=5, and the highest responder having 78.11 ± 5.6% positive moncytes, n=5 (Fig 5.3). . Thus, in the current study there was little sign of a high and low responder phenomenon at these doses. Although both Østerud et al., 1992 and Amirkhosravi et al., 1996 both observed a dual responder phenomenon to varying degrees, Østerud's 50 fold differences between high and low responders were in TF activity and not in TF antigen when measured by ELISA. Amirkhosravi et al., only observed a three fold difference (21-68%), in contrast to Østerud's 50 fold difference, but specifically in TF antigen. This is in contrast to the current study which demonstrates, at most, a 1.75 fold difference in TF positive monocytes between the highest and lowest responders. This may be due to differences in red cell lysis methodology, or to the fact that Amirkhosravi et al., used a 1 hour incubation time compared with 2 hours in the current study. Furthermore, Amirkhosravi used a directly conjugated monoclonal antibody to TF in contrast to our indirect staining method. Biological variation or assay variation may also have contributed to the variability seen at the 100ng/ml dose. Ultimately, the good

reproducibility of TF induction within subjects and between subjects at the 10μg/ml dose of LPS after 2 hours suggests that these conditions provide a good model for LPS induced TF antigen induction for use in subsequent experiments. Moreover, the observation that TF antigen varies little between individuals, coupled with Østerud's studies demonstrating that this is amplified to a 50-fold difference in TF activity between the highest and lowest responders, suggests that targeting TF antigen expression may be an efficient strategy for inhibiting the procoagulant effects of TF.

Finally, since subsequent studies are to be carried out in whole blood, it was necessary to characterize the association of GEM-91 ODN with monocytes in whole blood compared with freshly isolated PBLs. It has been shown that isolated PBLs, when incubated with FAM-labelled GEM-91, had more associated GEM-91 than leukocytes incubated in whole blood treated with GEM-91 (Figs 5.4a-d). At 5μM dose of GEM-91, the total cellular fluorescence was 15.86 ± 2.4 for cultured cells compared with 60.4 ± 19 for cells from whole blood incubations, a 4 fold difference. However, as in isolated cells, there was a similar heterogenous association of GEM-91 between different PBL subtypes, with monocytes having the greatest association, followed by neutrophils and finally lymphocytes. At GEM-91 concentrations of 0.1 and 1.0μM, ODN association was 10 fold less in whole blood than in isolated cells after a 4 hour incubation (GMF 12.3 ± 0.2 vs 131.33 ± 36.74 and 41.9 ± 1.3 vs 407.9 ± 68.8 n=4. At the highest GEM-91 dose tested (5μM), monocytes from whole blood incubations had 3.5 fold less associated ODN than isolated moncytes, (GMF=140.4 ± 11.8 vs 502.1 ± 86.2). In chapter 3, it was shown that in the presence of ODN, PBL subtypes bound and internalized GEM-91 over a period of 4 hours. Due to financial constraints it was not possible to measure intra- and extra-cellular uptake kinetics of the ODN again in whole blood. However, from the results of chapter 3, it can be assumed that any associated GEM-91 could feasibly be internalized. There are a number of possible reasons why cells isolated from whole blood incubated with GEM-91 may take up less ODN than cultured cells. Oligonucleotides may complex with plasma proteins in whole blood making less available to receptors on the cell surface. However, it has been documented that cationic lipids are not required for some ASODNs to have effect *in vivo* whilst in cultured cells the same ODNs require

liposomes to facilitate their effects (Bennett et al, 1992; Dean et al., 1994; Stepkowski et al., 1994). Therefore, it might be speculated that cultured cells, such as adherent monocytes can be artifactually activated by culture conditions and so may take up more ODN by the process of phagocytosis, resulting in intracellularly unavailable ODN. In contrast, monocytes in whole blood may take up less ODN but by the active process of receptor mediated endocytosis which may result in more bioactive ODN being available to its target. It is not known how much ODN is required for an antisense effect, but it is probable that even though whole blood monocytes take up less than their cultured counterparts, they may still possess enough intracellularly available ODN to have an antisense effect. Although anticoagulated, *ex vivo* whole blood is a far from perfect physiological model, it represents a much more realistic environment in which to study monocyte TF induction and its regulation, in so far as it has a full complement of PBL subtypes, red blood cells, platelets and plasma proteins all of which are involved in LPS induced TF expression and ODN uptake into cells.

In summary, this study has fully characterized the *ex vivo* whole blood model with respect to LPS induced TF antigen expression. It has been confirmed that this model of TF induction by LPS provides a suitably physiological system in which to study the antisense inhibition of monocyte TF by flow cytometry.

CHAPTER 6

INHIBITION OF TISSUE FACTOR INDUCTION ON MONOCYTES BY ANTISENSE TISSUE FACTOR OLIGODEOXYNUCLEOTIDES

6.1 Introduction

6.1.1 Genetic Therapies

The modification of the expression of specific mammalian genes for the purposes of creating novel therapies with fewer side effects has long been an aim of the medical profession. Over the past ten years advances have been made in gene targeting strategies based on the technique of homologous recombination. This technique has enabled genes to be altered on a routine basis producing cell lines and transgenic organisms with defined genotypes, (Capecchi *et al.*, 1989). Nevertheless, its general application is considerably constrained by the fact that it requires the transfection of large DNA constructs and so it is inefficient, expensive and restricted to a small number of cell types. Moreover, the practicalities of applying genetic manipulation techniques to gene therapy has been restricted by its low efficiency in that there is generally less than one modified cell in every 100 000 treated cells (Vasquez and Wilson, 1998). Parallel advancements in ODN technology offers several alternatives for modifying gene expression. ODNs which bind specifically to DNA (antigene ODNs) to RNA (antisense and ribozyme ODNs) and to proteins (aptamer ODNs) have been developed (Hanania *et al.*, 1995; Stull *et al.*, 1995). Antigene strategies focus primarily on gene targeting by homologous recombination or by triple helix forming oligodeoxynucleotides (TFOs). TFOs hybridize in the major groove of DNA by Hoogsteen or reverse Hoogsteen bonding and can disturb gene function by preventing the binding of transcription factors (Maher *et al.*, 1989; Helene, 1991a), by inhibiting duplex unwinding (Helene *et al.*, 1991b) or by inducing mutations in the target gene (Wang *et al.*, 1995; Ratajczak *et al.*, 1992). This non-vector based approach to manipulating gene function does not appear to be cell type specific but it is constrained by the need for polypurine/polypyrimidine target sequences (Gewirtz *et al.*, 1996). Antisense and ribozyme ODNs can be used to direct the destruction of specific RNAs or to prevent their translation (see later). Aptamer ODNs can be used to interfere with or selectively stimulate the functions of particular proteins.

ODNs are attractive molecules for therapeutic purposes because they can be easily synthesized in large quantities by automated procedures. Furthermore, antisense design does not require detailed knowledge of the structure of the target protein only of the target mRNA, both in terms of sequence and secondary structure. Finally ODNs can be designed to incorporate modified bases, backbone components and reactive chemical species to confer stability, increase binding or improve functionality such as in the case of phosphorothioate development (see chapter 3 for full discussion of ODN modifications). As mentioned in chapter three, many methods of delivering ASODNs to cells have been investigated including techniques whereby the ODN itself is modified, as in receptor ligand conjugation (Stephens and Rivers, 1997), or the target cells are treated to facilitate entry as in cell permeablization by electroporation (Bergan *et al.*, 1996; Flanagan *et al.*, 1997) or chemically by streptolysin (Giles *et al.*, 1995a; Bergan *et al.*, 1996; Flanagan *et al.*, 1997). Antisense technology is well advanced in its development as a genetic medicine with several antisense agents already in clinical trial, and will be discussed in more detail below.

6.1.2 Mechanisms of Action of ASODNs

There are multiple antisense mechanisms by which ASODNs may regulate the expression of target genes. The mechanism of action is dependent upon the design of the ASODN. Oligonucleotides designed to bind the pre-mRNA across the boundary between exons and introns (splice sites) may inhibit the splicing region. Kole *et al.*, 1996 have demonstrated that uniformly modified 2′-O-methyl phosphorothioate ODNs (ODNs with a modified sugar group), can alter the splicing of a thalassemic β-globin mRNA in mammalian cells. The ODN was used to mask a splice site so that an alternative site was used. Similarly, Hodges and Crooke 1995, have reported that 2′-O-methyl-phosphorothioate ODNs are capable of selectively blocking splicing of an adenovirus transcript. Alternatively, binding of ODNs to the target mRNA may inhibit gene expression through simple steric hindrance. The RNA-DNA duplex may simply block the RNA from physically interacting with cellular components required for translation of RNA into protein. For example, ODNs designed to bind the

translation initiation codon may compete with the ribosome for the site and thus inhibit translation. Several studies have demonstrated that ODNs are capable of inhibiting translation in this way in *in vitro* translation assays including experiments by Ochoa *et al.*, in 1961. However, it has proved more difficult to prove this mechanism in cell based assays. Alternatively, ODNs targeted to coding sequences downstream of the initiation codon may inhibit the assembly and translocation of the ribosomes along the mRNA. Baker *et al.*, 1997 utilised uniformly 2' modified ODNs which do not support RNase H activity (see later), to target the 5' terminus of ICAM-1 mRNA. These ODNs very effectively inhibited ICAM-1 protein expression by markedly changing the polysome profile of ICAM-1 mRNA shifting it from a higher molecular weight polysome pool to a lower molecular weight pool. Another mechanism of action of ASODNs is mediated by cleavage of the targeted mRNA by the endogenous ribonuclease RNase H. This enzyme is an endogenous ribonuclease which is located in the nucleus and in the cytoplasm and cleaves only hybridised mRNA/ODN duplexes. There is a large body of evidence suggesting that reduction or cleavage of the targeted mRNA is mediated by RNase H. This includes demonstration of a direct reduction in mRNA (Stewart *et al.*, 1996; Condon *et al.*, 1996), detection of appropriate cleavage products (Giles *et al.*, 1995b), and use of modified ODNs that do not support RNase H activity (Chiang *et al.*, 1991; Monia *et al.*, 1993).

6.1.3 Naked ASODNs versus Modified ASODNs

Many studies have been carried out both *in vitro*, in animal models and in clinical trials demonstrating the effects of ASODNs on disease. There is some controversy as to the best way of delivering ASODN to their target and these have been discussed already. They include encapsulation of the ASODN in liposomes or conjugation of the ASODN with cationic lipid to facilitate its entry into cells. This is thought to increase uptake of ASODNs and improve intracellular bioavailability. However, bearing in mind the cytotoxicity of cationic lipids, they are of little use in an *in vivo* setting. A study by Nestle *et al.*, 1994, has shown that cationic lipid is not required for uptake and inhibitory activity of ICAM-1 phosphorothioate ASODNs in

keratinocytes. Antisense phosphorothioate ODNs designed to hybridize to various regions of the ICAM-1 mRNA selectively influenced cultured keratinocyte ICAM-1 expression in response to gamma interferon (INF-γ). It was shown that keratinocytes rapidly internalized antisense compounds within 30-60 minutes with approximately 30% of the cells possessing positive nuclei. Using flow cytometry, they also demonstrated that IFN-γ induced ICAM-1 expression was reduced by 50% by the ASODN. Studies by Hartmann *et al.*, 1996, have demonstrated that a phosphorothioate ODN complexed with cationic lipid , designed to hybridise specifically to the translation start site of TNF mRNA at a concentration of 2μM inhibited TNF induction by LPS in freshly isolated monocytes by up to 79% compared with control ODNs. However, this group specify that certain steps in their experimental protocol were crucial for efficient inhibition of TNF by ASODNs including the serum-free incubation of ASODN and cationic lipid to prevent interaction between ASODN and serum proteins which can lower the ratio of available ODN (Srinivasan *et al.*, 1995). This suggests that the use of cationic lipid *in vivo* may not have the same effects as in a cell culture setting. Monia *et al.*, 1996 have demonstrated inhibition of tumour growth in a xenograft model by an antisense phosphorothioate ODN targeted to c-raf-1. A phosphorothioate ASODN targeted to specifically inhibit c-raf-1 kinase produced dose dependent inhibition of tumour growth over a range of concentrations (0.06-6mg/kg/day) for 21 days compared with mismatched ODNs (ODNs with a scrambled antisense sequence). Interestingly, cationic liposomes were required for the ASODN to have effects in cell culture studies but not in the *in vivo* experiments. This observation is consistent with other reports demonstrating that, although ODN transfection methods are required *in vitro*, these procedures are not required to elicit antisense effects *in vivo* (Bennett et al, 1992; Dean *et al.*, 1994; Stepkowski *et al.*, 1994). In addition, Pawlowska et al. 1998, have investigated the ability of a naked 16mer antisense phosphorothioate modified ODN to PAI-1 to increase fibrinolysis and modify experimental thrombosis in rats. They have shown that intravenous infusion of the ASODN (1-5mg/rat) produced a significant time and concentration dependent reduction in PAI-1 activity of blood plasma compared with control ODNs. This 20-30% inhibition was enough

to cause a profound delay in arterial occlusion time in rats treated with the ODN. This study also strongly suggests that cell permeation barriers *in vitro* may not be a problem *in vivo*.

6.1.4 Chloroquine

Although there have been reports demonstrating that naked phosphorothioate ODNs can enter peripheral blood leukocytes (Hartmann *et al.*, 1998, Pirruccello *et al.*, 1994), and experiments have been carried out showing that this uptake is, at least in part receptor mediated endocytosis (Beltinger *et al.*, 1995, Benimetskya *et al.*, 1997), controversy exists as to the fate of ODNs once inside the cell. It is thought that ODNs exert their effects either in the cytoplasm or in the nucleus. Although reports by Beltinger *et al.*, 1995 have detected phosphorothioate ODNs in both the cytoplasm and nucleus they have also detected a high concentration of ODN in cytoplasmic vesicles. How ODNs escape these vesicles and how rapidly this escape occurs is as yet unclear. Chloroquine (CQ) is a widely used anti-malarial drug. It is a lysosomatropic agent having its effect by accumulating in acidic food vacuoles and lysosomes within malarial parasites with subsequent disruption of vacuole functions. This is accomplished by raising the pH beyond the optimum required for parasite food digestion. The acidic food vacuole in the malarial parasite has been likened to the acidic phagolysosomes within mammalian cells (Ohkuma and Poole, 1978). It has been shown that CQ causes swelling of lysosomes in the malarial parasite *P. falciparum* (Yayon *et al.*, 1984), similar to the effect seen in mammalian lysosomes (Poole and Ohkuma 1981). Consequently, chloroquine has been shown to exert similar effects in leukocytes as in the malarial parasite. It can enter cells and accumulate in acid vesicles to a concentration estimated at 50nM as calculated on the estimated volume of the vesicular compartments (Erbacher *et al.*, 1996). Chloroquine is known to interfere with the endocytosis process and causes a number of effects that may influence the intracellular fate of ASODNs. Through its accumulation, it induces the neutralization and swelling of acidic vesicles leading to membrane destabilization.

In addition, by raising the pH of the lysosomes, it inhibits degradative enzymes which require an acidic environment (Ohkuma and Poole, 1978; Poole and Ohkuma, 1981; Maxfield 1982) and it inhibits vesicular transport thus reducing ligand delivery to the lysosomes (Strous *et al.*, 1985; Hedin and Thyberg, 1985; Stenseth and Thyberg, 1989). Chloroquine is also able to complex with DNA (Parker and Irvin, 1952). In the light of these properties, it is possible to envisage several mechanisms by which chloroquine could increase the efficacy of ASODNs. It could extend the effective intracellular half-life of the ODN by avoiding the lysosomal degradation pathway and reducing excretion or destabilize the endocytic compartments allowing more ODN to escape. Erbacher *et al.*, 1996 have demonstrated that addition of 100μM CQ for 3 or 4 hours increased the transfection efficiency of a human hepatoma cell line (HepG2 cells) by a plasmid DNA/lactosylated polylysine complex by neutralization and vacuolization of the endosomal and lysosomal vesicles. In addition, Stewart *et al.*, have demonstrated in a lung carcinoma cell line (A549 cells) that the inhibition of ICAM-1 expression by a phosphorothioate ASODN complexed with fucose conjugated poly-L-lysine was only observed in the presence of 100μM chloroquine. Therefore, we have examined the possibility of using CQ as a less cytotoxic way of improving ASODN action in human monocytes.

6.1.5 Inhibition of Monocyte TF Induction by ASODNs

To date, only one other group has investigated the use of ASODNs in the modulation of TF expression. Stephens and Rivers, 1997, have used the published DNA sequence of TF (Scarpati *et al.*, 1987) as a template to design a thirty base phosphorothioate ODN containing the rare tripeptide motif, Trp-Lys-Ser, which has been predicted as a functional motif involved in the interaction with serine proteases. They used CD14 receptor mediated endocytosis as a means of delivering the TF antisense ODN specifically to monocytes. They concluded that co-treatment of isolated monocytes with the antisense TF molecule and LPS resulted in an 80% suppression of TF activity compared with a control ODN. To our knowledge, there have been no reports testing ASODN effects on peripheral blood leukocytes in *ex vivo*

blood models. Furthermore, it is thought that phosphorothioate ASODNs may be more efficacious in an *ex vivo* whole blood setting compared with isolated monocytes in culture.

I have established a whole blood model to examine the induction of TF on monocytes, and using this model have investigated the effect of several ODNs on the LPS induced TF expression on monocytes in whole blood. I have tested the effect of Stephens and Rivers' ODN as well as a truncated version and a mismatched version of it on LPS induced TF in our system. In addition to the Stephens and Rivers ODN, I have designed an ASODN targeted to hybridize around the start site of translation of the TF mRNA and have tested this in my system when compared to the effects of GEM-91, an ODN which does not bind to the TF mRNA. Bearing in mind the apparent receptor mediated endocytosis mechanism of ODN uptake into cells, the effect of CQ on the efficacy of ASODNs to TF has also been examined.

6.2 Materials and Methods

6.2.1 Whole Blood Collection

Whole blood was collected from healthy volunteers by clean venepuncture with a 21 gauge needle into sterile vacutainers (Sarstedt, Germany) containing 3.2% trisodium citrate anticoagulant.

6.2.2 Effect of Antisense ODNs on TF Induction in Monocytes

Antisense ODN	Sequence	Description
AS1	AAAGCATTTGCTTTTCCAATCTCCTGACTT	ODN used by Stephens and Rivers (1997).
AS2	TTTGCTTTTCCAATCTCC	Truncated version of AS1
AS3	TGTTTTAACCATACCTCATTGGATCACTCT	Mismatched version of AS1
ASJM	GGGCCAGGCAGGGGTCTCCATGTCTACCAG	In-house designed ODN (hybridises at start-site of translation of TF mRNA).
ASGEM91	CTCTCGCACCCATCTCTCTCCTTCT	Control ODN not designed to hybridise to TF mRNA

TABLE 6.1. Antisense ODNs Tested

Aliquots (500µl) of citrated whole blood were placed into sterile polypropylene tubes and incubated directly with one of the above ODNs (1, 5 or 20µM final concentration as noted in a volume of 10µl) or 10µl sterile distilled water as a control, for 4 hours at 37°C at 95% air 5% CO_2. After 4 hours, 5µl LPS from *E. coli* O111:B4 (Sigma

Chemical Co.), final concentration 10μg/ml or 5 μl distilled water control, was added for 2 hours to induce TF expression. In control experiments, each of the antisense ODNs at each of the concentrations was added to whole blood and incubated for 4 hours to determine the effect of the ODNs alone on peripheral blood monocyte TF expression.

6.2.3 Effect of Chloroquine on Antisense Inhibition of LPS Induced TF on Monocytes

To examine the effect of the lysosomatropic agent chloroquine on the inhibition of TF induction by ASODNs, 10μM chloroquine (Sigma Chemical Co.) made up in culture medium, was added to whole blood aliquots in one of two regimes:

1. Chloroquine was added to a final concentration of 10μM concomitantly with the antisense ODNs for 4 hours before LPS addition.
2. Chloroquine was added to a final concentration of 10μM after the first 3 hours of antisense incubation, for 1 hour before LPS addition (Regime 2).

6.2.4 Immunostaining of Peripheral Blood Mononuclear Cells for TF Antigen in Preparation for FACS Analysis

After treatment, 100μl of whole blood was indirectly immunostained as described in chapter 2. 33μl of anti-tissue factor monoclonal antibody cocktail (30μg/ml stock solution) (kindly donated by Prof. T.E.Edgington, Scripps Research Institute, La Jolla, USA) in 67μl PBS was the primary antibody used. Whole blood 100μl aliquots were also added to tubes containing the same concentration of a negative control antibody (from an SV40 large T antigen not expected to bind to TF) supplied along with the anti-tissue factor monoclonal, or a phycoerythrin conjugated anti-CD14 monoclonal antibody (Sigma Chemical Co.) for monocyte recognition.

6.2.5 FACS Setup

Flow cytometry was performed using a FACScan (Becton Dickinson, Oxfordshire) running Cell Quest analysis software (Becton Dickinson). The monocyte population was identified by its forward and side scatter characteristics and routinely contained 95% CD14 positive cells. A total of 50000 cells was routinely collected which yielded approximately 2500 monocytes per 100µl blood sample. Monocyte cell surface TF was detected in the FL2 orange channel which detects the fluorescence emitted by phycoerythrin.

6.2.6 Data Analysis

Monocytes were gated as before by virtue of their forward and side scatter characteristics, and were typically 95% CD14 positive. The positive delineator (M1) was set as described in chapter 2 to include 3% of the cells stained with isotype control. All cells falling within this gate were subsequently deemed to be negative for TF. In addition, having examined the TF profile of cells treated with LPS two other markers were set: M2 was set to include cells with an intermediate fluorescence and M3 was set to include cells with a high fluorescence. This was necessary because some individuals had TF positive monocyte populations which expressed unusually high induced TF levels (See Fig. 6.1).

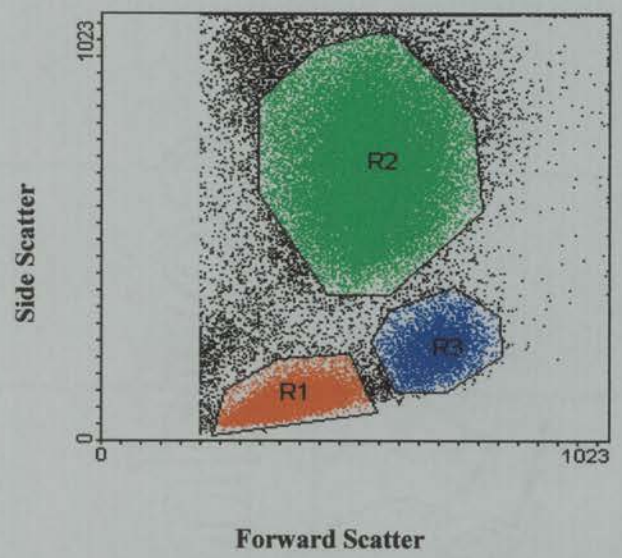
The percentage of the total monocytes which fell inside each of these markers was recorded for each sample. The M1, M2, M3 profile of the sample treated with the stimulatory LPS dose of 10µg/ml was used as a reference, and the percentage of cells within the corresponding markers in each test sample treated with ASODN was expressed as a **percentage change** over the reference sample (See Fig 6.1 for a worked example).

It is important to note that, as yet, it is not known whether an increase the number of TF positive monocytes, or an increase in the amount of TF antigen on each monocyte is a better measure of thrombogenicity. However, Amirkosravi et al., 1997 have shown that the percentage of TF positive monocytes as measured by flow cytomtery, is proportional to the monocyte procoagulant activity as measured by a functional

clotting assay. For the purpose of this study, the percentage of monocytes expressing TF was investigated and the ability of ASODNs to decrease the percentage TF positive monocytes in each of three defined categories of TF positivity.

6.2.7 Statistical Analyses

Except in the case of the TF inductions by ASODNs (where the TF levels were not high enough to have an M3 component), statistical analyses were performed on the M3 data, although data is graphically displayed on all three markers. This was as suggested by K. Peny of the University of Edinburgh Medical Statistics Department. All paired data was analysed by repeated measures analysis of variance followed by the Dunett's post test, where each data set is compared with one control data set, in this case the 10μg/ml treated sample. Where data could not be paired due to different sample sizes, a one way analysis of variance was carried out followed by a Dunett's post test. Although the data is represented graphically as percentage change over control, all statistics were carried out on raw data.



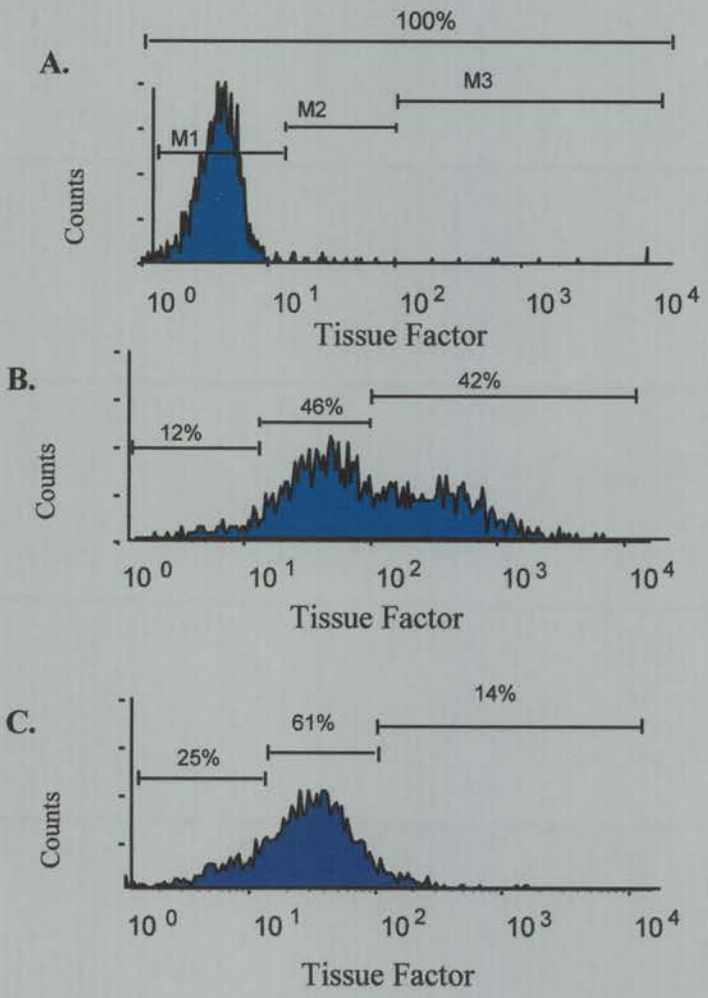


FIGURE 6.1 Marker settings for analysis of ASODN data and a worked example of the calculation. Cells coloured in blue are monocytes. Cells falling in the M1 gate are negative for TF. Cells falling in the M2 gate have intermediate TF positivity whilst cells in the M3 gate are highly TF positive. **A.** Monocytes from blood treated with 10μg/ml LPS have been immunostained with an isotype control mAb. **B.** Monocytes from blood treated with 10μg/ml LPS have been immunostained with a TF mAb and a TF induction is shown. **C.** Monocytes from blood treated with 10μg/ml LPS + ASODN. A 66% inhibition of TF positive monocytes is shown. (42-14)/42)*100=66%

6.3 Results

6.3.1 M1, M2, M3 Profile of TF-Induction on Monocytes

Subject	%M1	%M2	%M3
1	15.37	31.07	54.07
2	18.45	60.21	22.89
3	31.31	34.38	31.39
4	24.73	64.83	10.97
5	15.33	66.07	18.77
6	12.38	46.42	42.79
7	20.33	70.17	10.67
8	19.1	70.64	10.26
Mean	**19.6**	**55.5**	**25.2**

Table 6.2 TF Positive (M1, M2, M3) Profile of Monocytes Treated with 10μg/ml LPS for 4h (n=8). Numbers represent the % positive cells within each of the three markers. It can be seen that certain individuals have a higher proportion of monocytes in M3 underlining the need for three marker analysis.

6.3.1 Effect of ASODNs alone on Peripheral Blood Monocytes

Three of the ASODNs tested actually induced TF on monocytes in whole blood. AS1, the Stephens and Rivers oligo, AS2 its truncated version and AS3 its mismatched version at final concentrations of 1 and 5μM all produced significant increases in the percentage of monocytes falling into the M2 marker when compared with antisense untreated cells (4.44 ± 0.85 % vs 60 ± 7.152 % for 1μM and 59 ± 3.52 % for 5μM AS1; 22.8 ± 4.9 % for 1μM and 45 ± 4.9 % for 5μM AS2, and finally, 76.42 ± 2.13 % for 1μM and 71.9 ± 6.63 % for 5 μM AS3. ASJM and ASGEM-91 did not significantly induce TF with respect to untreated blood (Table 6.2, Fig 6.2)

ASODN	% Monocytes in M2	n	p value
0AS	4.4 ± 0.85	8	
1μM AS1	60 ± 7.12	6	<0.01
5μM AS1	59 ± 3.52	6	<0.01
1μM AS2	22.8 ± 4.9	6	<0.01
5μM AS2	45.4 ± 4.9	6	<0.01
1μM AS3	76.42 ± 2.13	4	<0.01
5μM AS3	71.9 + 6.63	4	<0.01
1μM ASJM	2.9 ± 0.72	8	NS
5μM ASJM	5.1 ± 1.2	8	NS
20μM ASJM	5.4 ± 1.3	8	NS
1μM ASGEM	4.2 ± 2	8	NS
5μM ASGEM	7.62 ± 1.41	8	NS
20μM ASGEM	5 ± 1.23	8	NS

TABLE 6.3. TF Induction By ASODNs

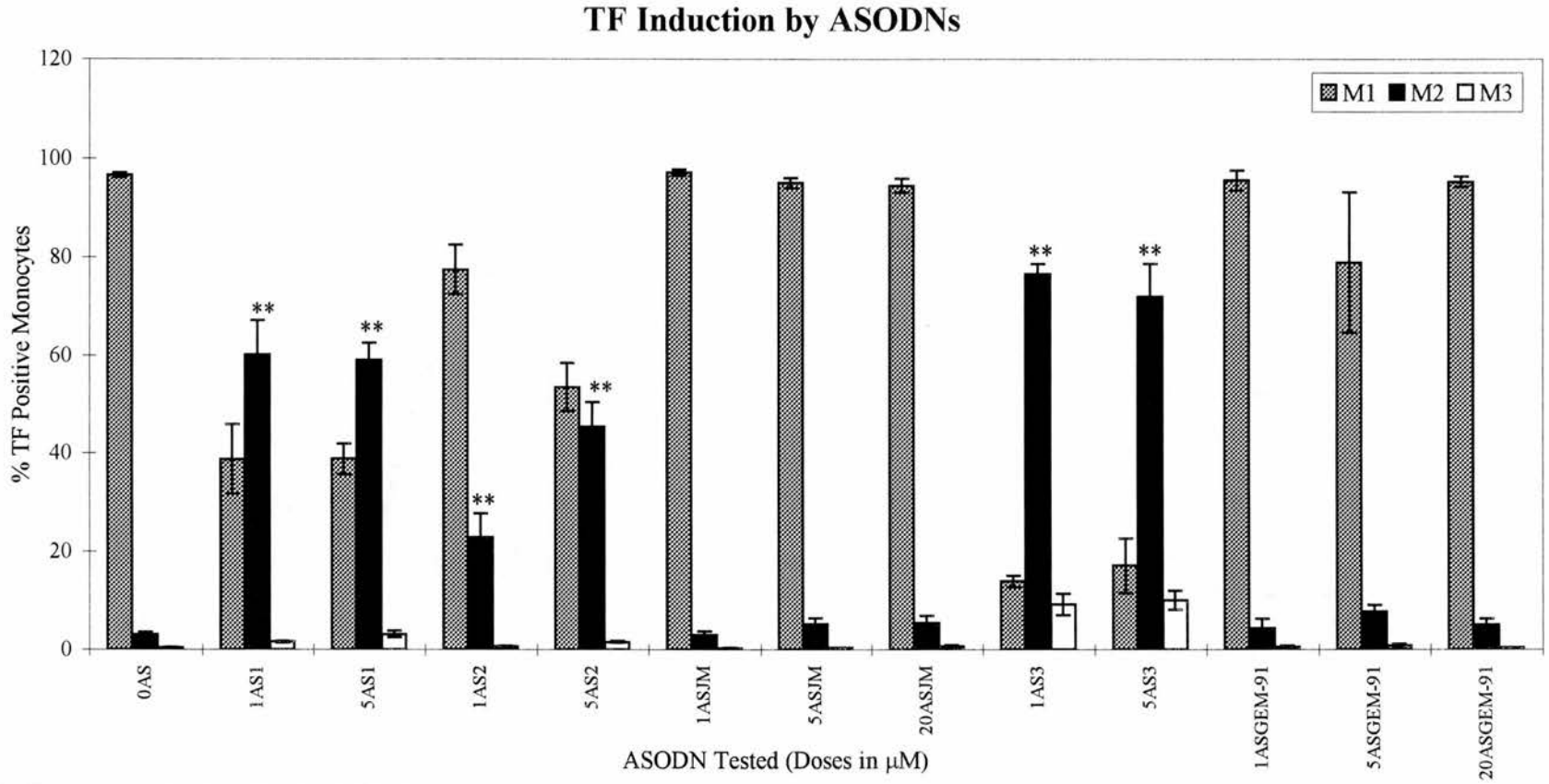


FIGURE 6.2 Induction of TF by ASODNs.

Whole blood was incubated with distilled water (0AS) or one of 2 concentrations of each ASODN for 4 hours. bars represent the mean percentage of monocytes in each gate +/- sem. It can be seen that AS1, AS2 and AS3 all caused a significant increase in the percentage of TF positive monocytes in the M2 marker when compared with ASODN untreated blood., whereas ASJM and its control GEM-91 did not. ** $p<0.01$ n=6 for AS1, AS2, AS3, GEM-91, n=8 for ASJM .

6.3.2 Effect of AS1, AS2, AS3 on LPS Induced TF Expression on Peripheral Blood Monocytes

AS1, the ASODN to TF designed by Stephens and Rivers significantly inhibited the TF induction by LPS. The mean percentage change in TF positive monocytes treated with 1μM AS1 falling within the M3 marker was 42.2 ± 9.544 % inhibition ($p < 0.01$) (Fig. 6.3) with respect to cells treated with LPS alone. Neither the truncated version nor the mismatched version caused a significant inhibition in the cells falling in M3.

6.3.3 Effect of ASJM and AS GEM-91 on LPS Induced TF Expression on Peripheral Blood Monocytes

ASJM, the in -house designed ASODN to TF designed to hybridise around the start site of translation of the TF mRNA at neither 1μM, 5μM or 20μM significantly inhibited the TF induction by LPS. The percentage of monocytes falling in the M3 marker did not significantly change when the blood was pre-treated with this ASODN compared with that pre-treated with distilled water (Fig 6.4).

6.3.4 Effect of Chloroquine Alone on TF Induction

Chloroquine alone did not significantly induce TF at either a 80μM or 10μM dose when compared with LPS induction of TF. However, at 80μM final concentration, chloroquine alone inhibited TF induction by approximately 70% after a 2h co-incubation with LPS, and 60% after a 4h co-incubation with LPS (53.4 ± 5.5% TF positive monocytes vs 15.37 ± 4.04 % after 2h co-incubation, 62.03 ± 6.06% TF positive monocytes vs 23.58 ± 2.35 % after a 4h incubation). However, at a final concentration of 10μM which is more in the range therapeutically tolerated by malaria patients, chloroquine alone did not significantly inhibit LPS induced TF after a 4h co-incubation with LPS (62.03 ± 4.91 % positive monocytes treated with LPS vs 65.28 ± 4.91 % positive treated with LPS+10μM chloroquine. It was, therefore, decided that a 10μM dose of chloroquine could be used in conjunction with the ASODN panel

in an attempt to improve their bioavailability without reducing TF induction on its own (Fig 6.5).

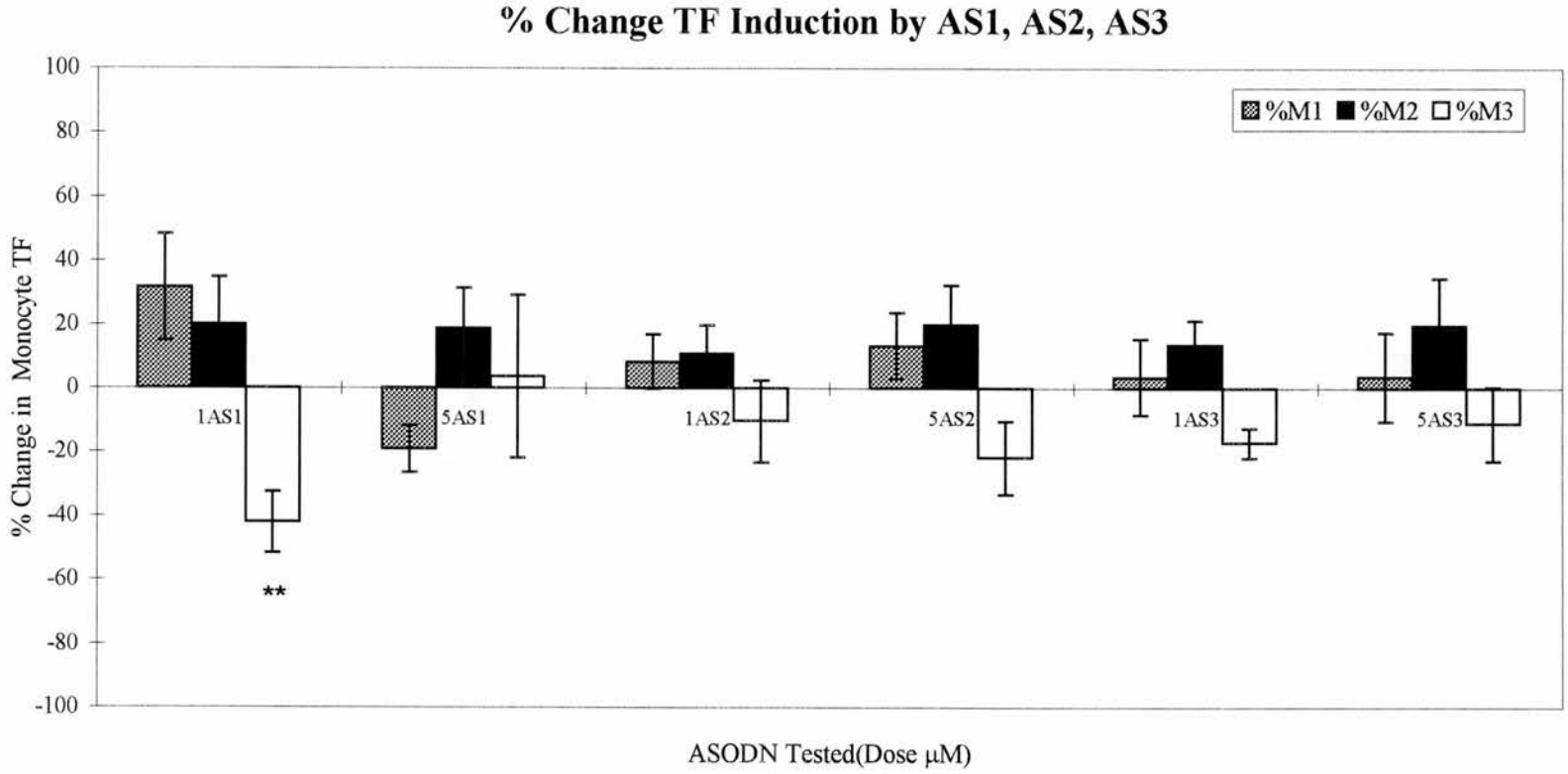


FIGURE 6.3 Percentage Change in Number of TF Positive Monocytes due to AS1, AS2 and AS3.
Whole blood was incubated with 2 concentrations of each ODN(1 and 5μM) for 4 hours before being stimulated with 10μg/ml LPS. Bars represent the mean percentage change +/- sem (n=6) in the percentage of monocytes in M1, M2 and M3 with ASODNs when compared with LPS stimulated whole blood not pre-treated with ASODN. Positive bars indicate a percentage increase in monocytes in that marker. Negative bars indicate a percentage inhibition of TF positive monocytes in that marker. It can be seen that at the 1μM dose, AS1 (Stephens and Rivers ASODN to TF) produced a significant inhibition of TF positive monocytes in the M3 marker, **$p<0.01$ n=6 (Repeated Measures ANOVA followed by Dunnett's Post test)

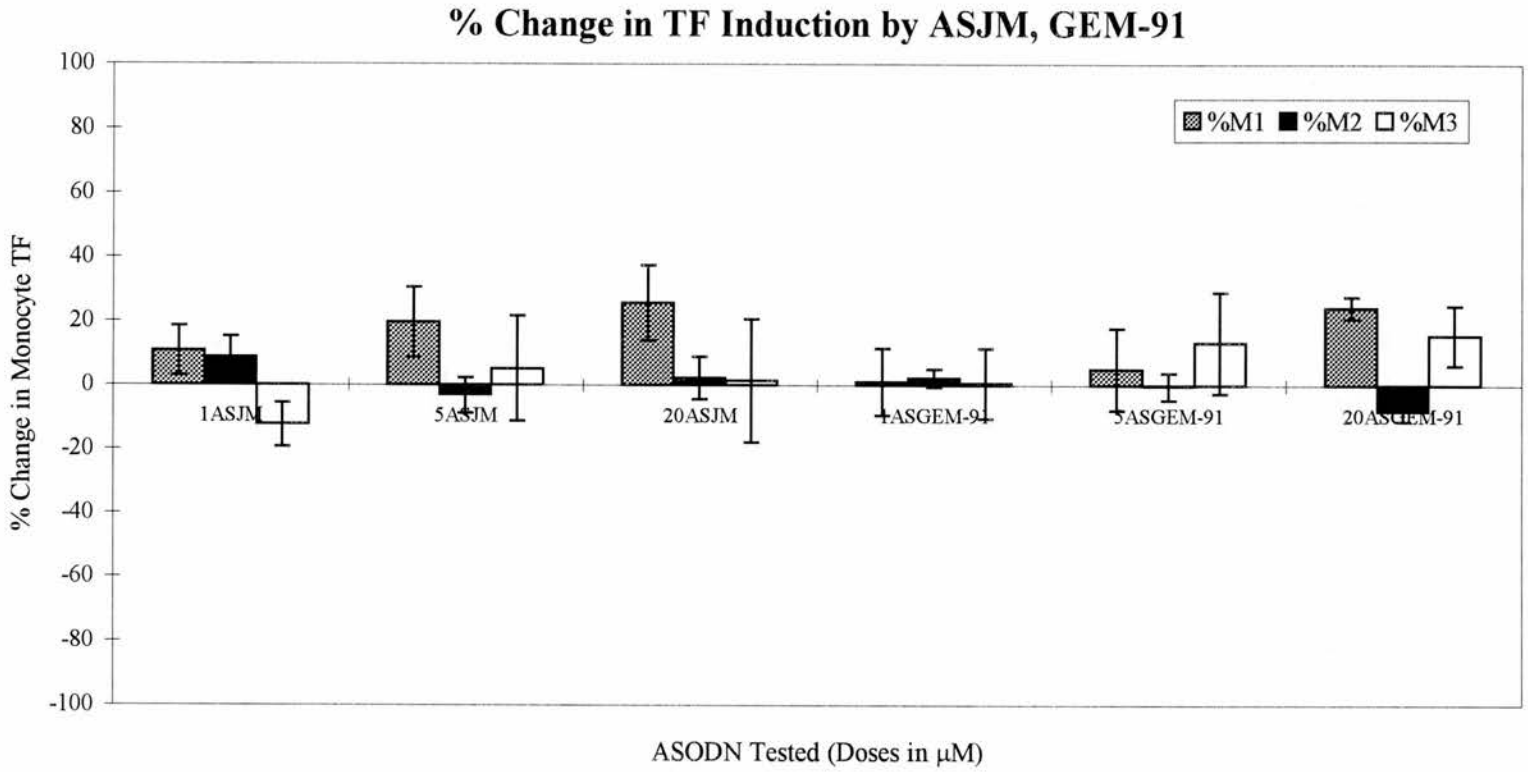


FIGURE 6.4 Percentage Change in Number of TF Positive Monocytes due to ASJM and GEM-91
Whole blood was incubated with 3 concentrations of each ODN(1,5 and 20μM) for 4 hours before being stimulated with 10μg/ml LPS. Bars represent the percentage change in the percentage of monocytes in M1, M2 and M3 with ASODNs when compared with LPS stimulated whole blood not pre-treated with ASODN. Positive bars indicate a percentage increase in monocytes in that marker. Negative bars indicate a percentage inhibition of TF positive monocytes in that marker. It can be seen that neither of the ASODNs tested under these conditions produced a significant inhibition of TF positive monocytes.

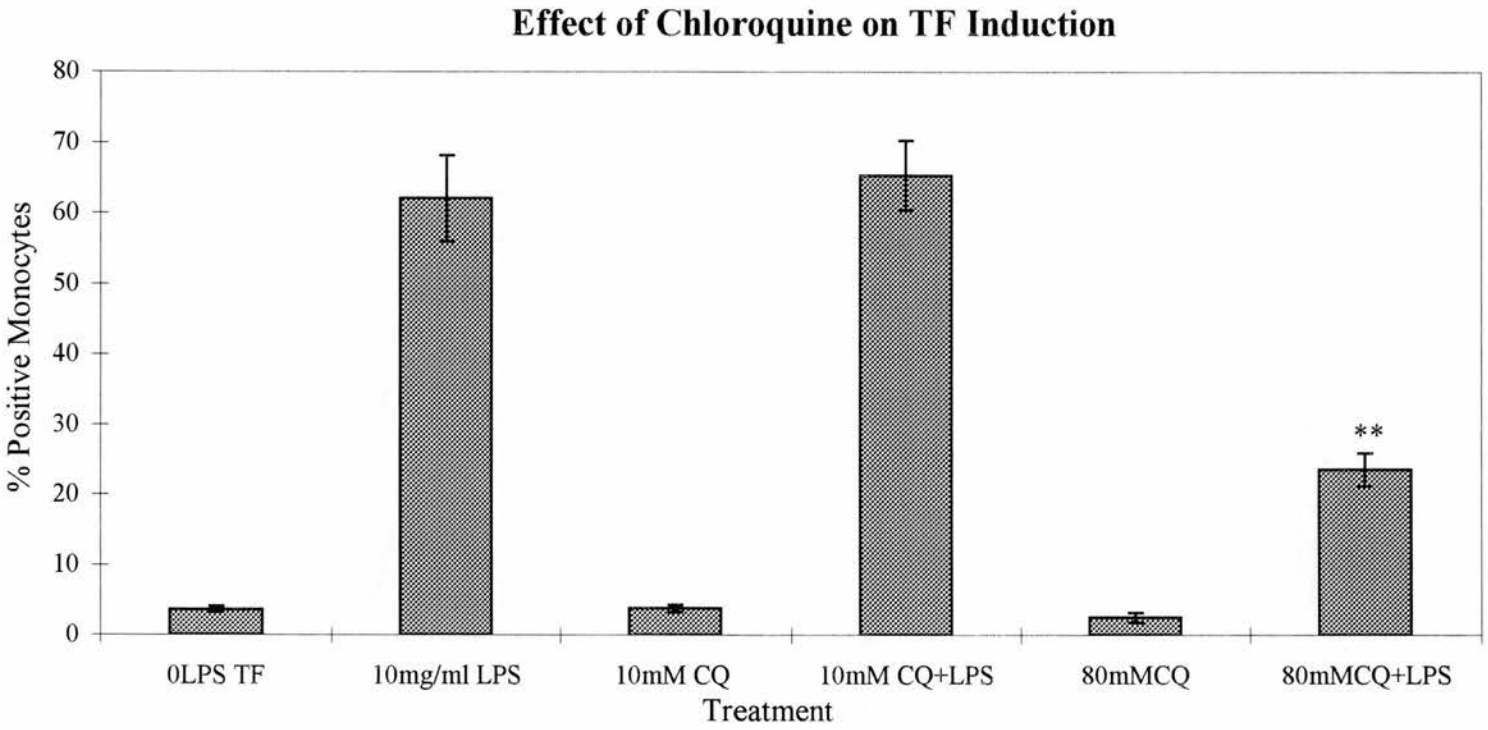


FIGURE 6.5 The Effect of CQ on TF Induction in Monocytes. Whole blood aliquots were treated with distilled water, 10mg/ml LPS, each of 2 concentrations of CQ, or were co-incubated with LPS and CQ. It can be seen that at 10mM or 80mM CQ did not induce TF. However, at a concentration of 80mM CQ inhibited TF induction by LPS whilst at 10mM it had no effect. This allowed a concentration of 10mM to be used in subsequent inhibition studies. Bars represent mean % positive monocytes +/- sem, n=8.

6.3.5 Effect of Chloroquine on AS1, AS2, AS3 Modulation of LPS Induced TF Expression

It was postulated that the lysosomatropic agent chloroquine, at a concentration of 10μM might increase the intracellular bioavailability of the ASODN by bursting cytoplasmic endosomes thus releasing more ASODN into the cytoplasmic compartment. On examining the percentage change in the proportion of monocytes falling inside the M3 marker, it appears that addition of chloroquine at a final concentration of 10μM significantly improved the inhibitory effects of AS1 and AS2, (ASODNs to TF) but not AS3 (mismatched version of AS1). Pre-incubation of blood with 1 or 5 μM AS1, or 1 or 5μM AS2 with 10μM chloroquine added simultaneously for 4h produced significant mean % changes in the proportion of cells falling into M3; 45.5 ± 6.7 %, 42.5 ± 14.3, 32 ± 8.9 %, 43.1 ± 9.4 % inhibitions respectively when compared with samples treated with LPS alone (n=6; $p < 0.01$). Chloroquine did not significantly improve the inhibitory effect of the mismatched control AS3.

ASODN	Mean % Inhibition M3	p	Mean%Inhibition M3 +CQ	p	n
1μM AS1	42.2 ± 9.5	<0.01	45.5 ± 6.7	<0.01	6
5μM AS1	-3.65 ± 25.6	ns	42.5 ± 14.3	<0.01	6
1μM AS2	10.5 ± 12.7	ns	32.0 ± 8.9	<0.01	6
5μM AS2	21.9 ± 11.5	ns	43.1 ± 9.4	<0.01	6
1μM AS3	17.3 ± 4.7	ns	28.0 ± 7.18	ns	6
5μM AS3	11.3 ± 11.7	ns	23.0 ± 6.81	ns	6

TABLE 6.4 Inhibition of Monocyte TF by AS1, AS2 and AS3 with and without the addition of CQ. Raw data were analysed by a repeated measures analysis of variance followed by a Dunnett's post test, where each group was compared with a control, in this case the 10μg/ml LPS treated group. Negative values represent an induction rather than a inhibition.

6.3.7 Effects of Chloroquine Administered in a Different Regime on ASJM and ASGEM-91 inhibition of TF induction

It was postulated that the effects of chloroquine may be more pronounced on the blood treated with ASJM if it was administered in a different regime. Blood was pre-incubated with ASODN for 4 hours and chloroquine was added for the last hour of the pre-incubation. This regime did not significantly improve the performance of the ASODNs with only the 1μM dose producing a significant inhibition; 41 ± 9% inhibition with respect to samples not treated with ASODNs.

ASODN	Mean % Inhibition M3	p	Mean%Inhibition M3 +CQ	p	Mean%Inhibition M3 +CQ Reg2	p	n
1μM ASJM	12.5 ± 6.9	ns	37.0 ± 7.7	<0.05	41.9 ± 9.01	<0.01	8
5μM ASJM	-5.5 ± 5.55	ns	13.9 ± 12.4	ns	21.5 ± 11.64	ns	8
20μM ASJM	-1.36 ± 6.64	ns	30.9 ± 12.1	<0.05	32.0 ± 17.65	ns	8
1μM GEM-91	-0.51 ± 2.9	ns	22.9 ± 6.1	ns	-7.24 ± 27.87	ns	5
5μM GEM-91	-13.32 ±4.27	ns	10.9 ± 7.8	ns	10.6 ± 20.54	ns	5
20μM GEM-91	-15.55 ± 2.99	ns	1.75 ± 4.75	ns	-4.92 ± 11.92	ns	5

TABLE 6.5 Inhibition of Monocyte TF by ASJM, GEM-91 with and without the addition of CQ under Regime 1and Regime 2. Meaned data were analysed by a one-way analysis of variance followed by a Dunnett's post test, where each group was compared with a control, in this case the 10μg/ml LPS treated group. Negative values represent an induction rather than a inhibition.

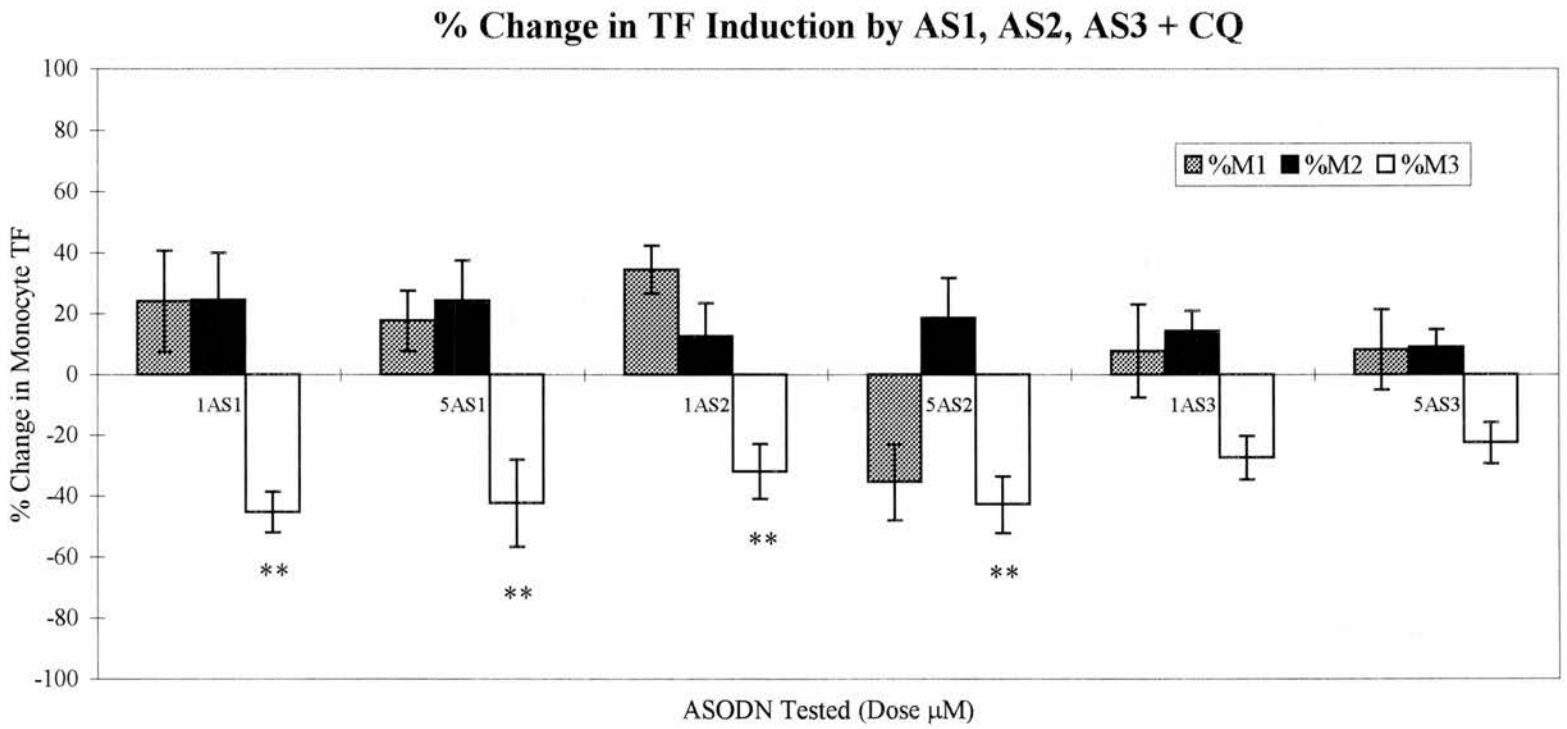


FIGURE 6.6 Percentage Change in Number of TF Positive Monocytes due to AS1, AS2 and AS3 + 10μM CQ.
Whole blood was coincubated with 2 concentrations of each ODN(1 and 5μM) plus 10μM CQ for 4 hours before being stimulated with 10μg/ml LPS. Bars represent the mean percentage change (+/- sem; n=6) in the percentage of monocytes in M1, M2 and M3 with ASODNs when compared with LPS stimulated whole blood not pre-treated with ASODN. Positive bars indicate a percentage increase in monocytes in that marker. Negative bars indicate a percentage inhibition of TF positive monocytes in that marker. It can be seen that at the 1μM dose, AS1 (Stephens and Rivers ASODN to TF) and AS2 (Truncated version of AS1) produced a significant inhibition of TF positive monocytes in the M3 marker, whereas AS3 (Mismatched Control ODN) did not. **$p<0.01$ n=6 (Repeated Measures ANOVA followed by Dunnett's Post test)

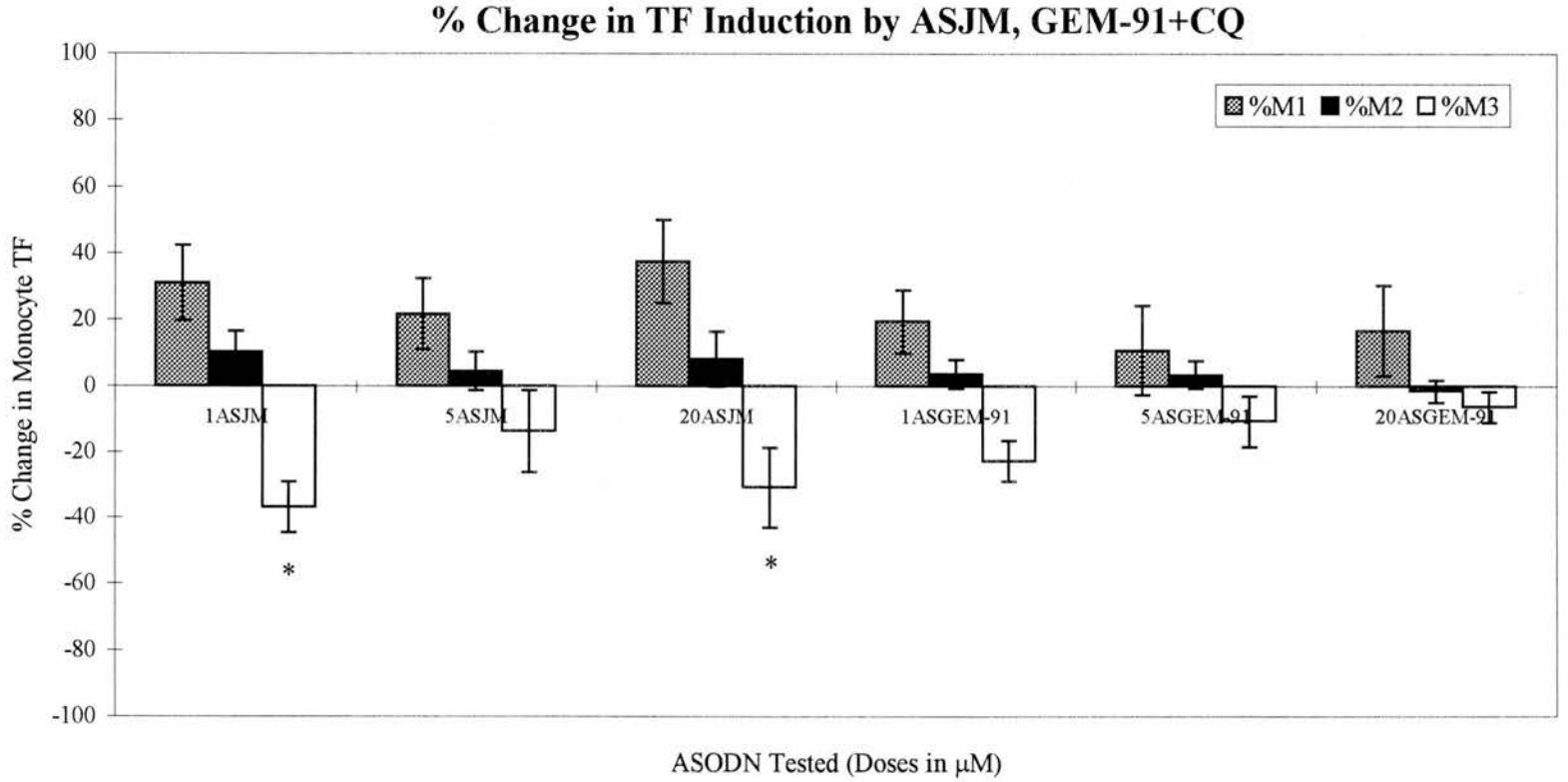


FIGURE 6.7 Percentage Change in Number of TF Positive Monocytes due to ASJM and GEM-91+10μM CQ.
Whole blood was incubated with 3 concentrations of each ODN(1,5 and 20μM) for 4 hours before being stimulated with 10μg/ml LPS. Bars represent the percentage change in the percentage of monocytes in M1, M2 and M3 with ASODNs when compared with LPS stimulated whole blood not pre-treated with ASODN. Positive bars indicate a percentage increase in monocytes in that marker. Negative bars indicate a percentage inhibition of TF positive monocytes in that marker. It can be seen that ASJM at the 1 and 20μM doses produced a significant inhibition of the Percentage of TF positive monocytes in the M3 marker, whereas GEM-91 the control ASODN did not. Meaned data were analysed by a one way ANOVA followed by a Dunnett's post test. *$p<0.05$, n=6 for GEM-91, n=8 for ASJM.

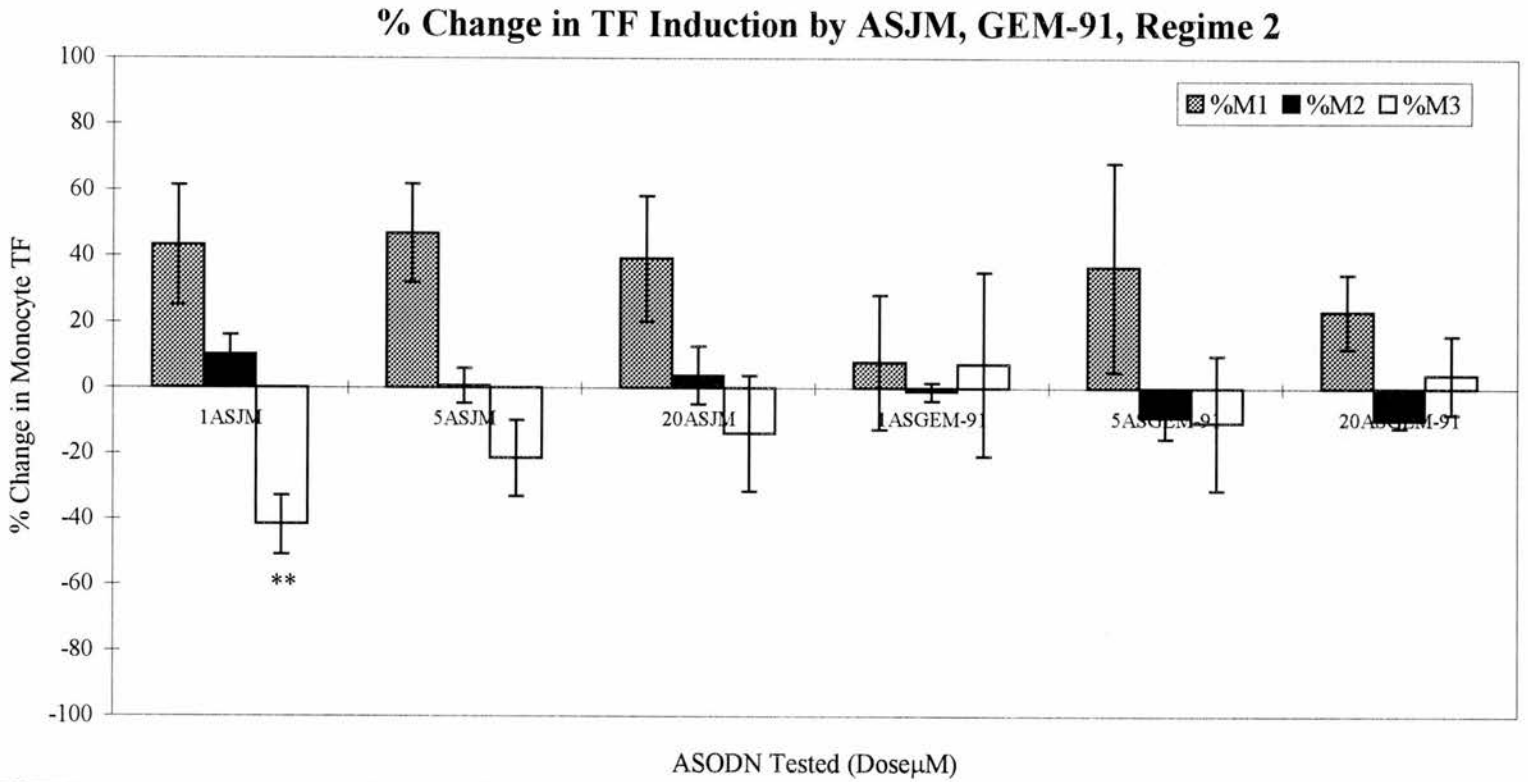


FIGURE 6.8 Percentage Change in Number of TF Positive Monocytes due to ASJM and GEM-91 + 10μM CQ Administered in Regime 2. Whole blood was incubated with 3 concentrations of each ODN(1,5 and 20mM) for 4 hours before being stimulated with 10μg/ml LPS. 10μM CQ was added for the final hour of ODN incubation only. Bars represent the percentage change in the percentage of monocytes in M1, M2 and M3 with ASODNs when compared with LPS stimulated whole blood not pre-treated with ASODN. Positive bars indicate a percentage increase in monocytes in that marker. Negative bars indicate a percentage inhibition of TF positive monocytes in that marker. It can be seen that ASJM at the 1μM dose produced a significant inhibition of TF postive monocytes in the M3 marker, whereas the same concentration of GEM-91 did not. Mean values were analysed by a one-way ANOVA followed by a Dunnett's post test **$p<0.01$, n=8 for ASJM, n=6 for GEM-91.

6.4 Discussion

It has been determined so far in this study that the monocyte appears to be a suitable target cell for antisense therapy in so far as it internalizes ODNs more efficiently than the other PBL subtypes. Bearing in mind that monocytes are the only PBLs capable of expressing TF, the experiments outlined in the chapter set about investigating the potential of ASODNs to TF at inhibiting the LPS-induced expression of this protein in a whole blood model. Initially, an ASODN directed to the start site of translation of the TF mRNA was designed. However, during the course of my preliminary studies, Stephens and Rivers, 1996 reported the inhibition of TF activity (but not antigen), using an ASODN to TF designed by them. Subsequently, their ODN was included in the current studies as a comparison to the in-house designed ASODN to TF.

6.4.1 Induction of TF by ASODNs

Initially, the effect of an incubation with each of the ASODNs alone on whole blood was investigated. It was shown that AS1, the ASODN to TF designed by Stephens and Rivers, AS2, its truncated version and AS3 its mismatched version, all significantly induced TF antigen on monocytes in whole blood after a 4 hour incubation as measured by flow cytometry. AS1, and AS3 its mismatched version, induced the most TF, the percentage of monocytes falling in the M2 marker was 60 ± 7.12% and 59 ± 3.52% with 1μM, and 5μM AS1 respectively and 76.42 ± 2.13 and 71.9 ± 6.63% with 1μM and 5μM AS3 respectively ($P<0.01$) when compared with 4.4 ± 0.85% for AS untreated blood. The truncated ODN, AS2, was less potent, with 22.8 ± 4.9 and 45.4 ± 4.9% monocytes falling in the M2 marker with 1μM and 5μM AS2 respectively. In contrast, neither ASJM, the in-house designed ASODN, nor GEM-91 significantly induced TF antigen on monocytes (Fig 6.2., Table 6.2) It appears, therefore, that the TF inducing effect of the ODNs is not particular to phosphorothioates; as is the case for other non-specific cardiovascular effects seen in *ex vivo* whole blood (Shaw *et al.*, 1997). This group have shown that

phosphorothioates cause a decrease in partial thromboplastin time that is not sequence specific, but rather is specific to the phosphorothioate linkage. The TF induction by phosphorothioates seen in the current study may, however, be due to a combination of ODN sequence and length effects. Although AS2 (18mer) the truncated version of AS1(30mer), was less potent at inducing TF than AS1, the two longer ODNs ASJM (30mer) and GEM-91(25mer), did not significantly induce TF. However, AS2, ASJM and GEM-91 have in common that they contain fewer adenosines than AS1 and A3. This observation suggests that the presence of adenosine may predispose the ODN to TF induction. This could feasibly occur by interaction of the ODN with the LPS receptor CD14, setting into motion intracellular signals culminating in transcription of the TF gene. It has been shown by Fan *et al.*, 1991, that ligands of the Mac-1 receptor are capable of enhancing 2 to 8 fold the expression of TF. They have postulated that engagement of Mac-1 by complementary ligands results in transduction of cellular signals that enhance the expression of TF expression on monocytes. As discussed in chapter 4, Benimetska *et al.*, 1996 have shown that Mac-1 is a cell surface receptor for ODNs. It might be speculated that binding of certain ODNs to Mac-1 might induce intracellular signalling events to enhance any basal TF expression occurring as a result of incubation conditions.

6.4.2 Inhibition Studies

The potential of 5 ASODNs at inhibiting LPS induced TF antigen expression on monocytes in whole blood was then investigated. It was shown that incubation of whole blood with AS1 at a concentration of 1μM significantly inhibited the TF induction of TF by LPS producing a 42.2% reduction in the number of cells strongly positive for TF, ie those cells falling in the M3 marker (Fig 6.3) ($p<0.01$, $n=6$) when compared with the truncated version, and the mismatched version of the ODN. Neither ASJM nor GEM-91 produced a significant inhibition in TF induction by LPS (Fig 6.4). Stephens and Rivers have investigated the effect of AS1 as well as a mismatched, a sense and an irrelevant control ODN, all of which were linked to a CD14 monoclonal antibody conjugated with poly-L-lysine carrier complex, on TF

activity in freshly isolated, lysed monocytes. They concluded that co-incubation of the ASODN to TF, but not the control ODNs, with LPS resulted in a marked suppression of TF biological activity by 80.4 ± 2.2% when compared to monocytes treated with LPS in the absence of TF ASODN. They also conclude that the CD14-poly - L-lysine carrier system is required for the ODN to have its effect. In the current study, I have shown a significant inhibition of TF antigen by a naked version of Stephens and Rivers ASODN (AS1), at the 1μM dose, but not at the 5μM dose. This may be due to the fact that the ASODN is already at its maximal dose at 1μM and any further increase in the dose of ODN may cause increased TF induction by the ODN to interfere with the inhibition. In their study, Stephens and Rivers do not attempt to measure TF antigen, and so it is not certain whether the ASODN is reducing TF activity by actually decreasing translation of the protein. I have attempted to improve the efficacy of the ODNs tested by incubating them in conjunction with the lysosomatropic anti-malarial agent chloroquine. Chloroquine has been shown to improve intracellular bioavailability of ODNs (Loke *et al.*, 1989) possibly by inhibition of intracellular recycling of ODNs or by releasing ODN from intracellular acid vesicles. At a 10μM dose CQ did not on its own inhibit TF induction and so this dose was chosen for co-incubation experiments (Fig 6.5). It has been shown that co-incubation of 10μM CQ with 1 or 5 μM AS1 or AS2 for 4 hours before LPS stimulation, caused a significant inhibition in TF antigen induction. Incubation with 1 and 5μM AS1 produced a 45.5 ± 6.7% and 42.5 ± 14.3% inhibition in the percentage of monocytes falling in the M3 marker compared with samples treated with LPS alone. Similarly, co-incubation of whole blood with 1 and 5μM AS2, the truncated ODN, resulted in a 32 ± 8.9 and 43.1 ± 9.4% reduction in the percentage of monocytes strongly positive for TF. Pre-incubation with the control mismatched ODN AS3 did not result in an inhibition which reached significance (Fig.6.6, Table 6.3). However, it was evident that in some individuals this control ASODN did cause some inhibition of TF antigen induction, especially in the presence of CQ. This suggests that the effects seen with these ODNs are not purely antisense effects but are enhanced by non-specific interactions.

The in-house designed ODN ASJM, which was designed to hybridize around the start site of translation of the TF mRNA gave less promising results in the absence of CQ than AS1. Without the addition of CQ, no significant inhibition of TF was seen any of the three doses tested. It was clear that some individuals did respond, but the inhibitions seen were not reproducible and so did not reach significance. In the presence of CQ, the effect of ASJM was improved, resulting in a significant reduction in the percentage of strongly TF positive monocytes (Fig 6.7, Table 6.5). It was thought that the CQ might further improve the inhibitory effects of the ODNs if administered in a different regime. When CQ was added to the whole blood for the final hour of ODN pre-incubation only, there was a slight improvement in ASJM effect at the 1μM dose, resulting in a 41 ± 9% inhibition in TF (Fig 6.8, Table 6.6). This improvement was not seen at the higher doses. In addition, GEM-91 which was used as a control ODN in this part of the study did not elicit any significant inhibition of TF. It is, therefore clear that CQ does help the efficiency of ODN action. The mechanism for this cannot be determined from this study but is likely to be a result of CQ bursting intracellular vesicles, thus releasing any sequestered ODN. As CQ is already used therapeutically as an antimalarial agent at doses around 10μM, this study suggests that it might be feasible to administer CQ with ASODNs as less toxic alternative to cationic lipids for increasing intracellular bioavailability.

The most important observation in this study is that although each of the ODNs tested significantly inhibited TF antigen induction under certain circumstances, there was never a total inhibition by any of the ODNs and the inhibitions seen were not dose dependent. There are a number of possible reasons why the efficacy of the ODNs was so erratic. It is possible that the ODNs were not consistently reaching their intracellular targets. It has been documented that ODNs taken up by receptor mediated endocytosis often become sequestered in intracellular acid vesicles (Loke *et al.*, 1989; Bennett *et al.*, 1992) meaning that although they find their way into the cell, they do not reach their target mRNA. Although we have shown that CQ improves the efficiency of ASODNs in some individuals and at certain ASODN doses, it does not consistently do so. Secondly, it is possible that the ASODN is reaching the proximity of the mRNA to be inhibited, but that the precise target site is inaccessible.

The internal structures of target mRNAs and their associations with cellular proteins create physical barriers, which render most potential binding sites inaccessible to ASODNs. For Watson-Crick binding to occur, nucleic acid drugs must be complementary to exposed regions of their target mRNA. In the current study the start site of translation of the TF mRNA has been targeted which theoretically should be accessible for ODN binding. However, other sequences in the mRNA may be better ODN binding regions and so mRNA targeting is essentially a hit or miss process. Currently, effective nucleic acid drugs are being selected from large pools of candidate sequences. Although the microarray technique used by large drug companies, whereby multiple sequences are screened to find the best match for an antisense molecule, improves the binding efficiency of ASODNs, the ability to determine which regions of a given mRNA molecule are accessible has been a significant impediment to the design of effective ASODNs. Recently, Peng Ho *et al.*, 1998 have described an RNA mapping technique to identify accessible sites on mRNA molecules, whereby the RNA transcript of interest is probed with a library of semi-random chimeric ODNs. Regions of the RNA accessible for hybridization with members of the ODN library were cleaved using RNase H. Subsequent sequencing of the newly created RNA ends using primer extension analysis identified potentially effective sites for the targeting of antisense ODNs. The expense of techniques like this is likely to limit them to large corporations.

In the current study, control ASODNs were used in an attempt to determine whether the effects of the ODNs tested were true antisense effects or were due to non-specific ODN effects. The observations described in chapter 4 show that ODNs are capable of causing a reduction in monocyte cell surface CD14 and Mac-1 receptors, both of which are receptors for LPS and are involved in LPS induced stimulation of TF expression (Ingalls and Golenbock, 1995; Fenton and Golenbock 1998). Although neither the control ODN used in conjunction with Stephens and Rivers antisense molecules, namely a mismatched version of the true ODN, nor the GEM-91 ODN which was used as a control ODN in studies with the in-house designed ASJM produced any significant inhibition of TF it was clear that in some individuals a degree of inhibition, albeit irreproducible, occurred (Figs 6.3-6.8). In addition, binding of the

ODNs to the TF antigen itself may have inhibited antigen detection by the monoclonal antibody, resulting in an apparent reduction in the protein. Taken along with the apparent erratic inhibitions seen with some of the ASODNs tested, and the lack of extensive control ODNs used it is impossible to conclude that any effects seen are true antisense effects. Non-specific antisense effects have been well documented throughout antisense literature and sometimes produce surprisingly therapeutic results. Nevertheless, these non-specific effects have hampered the ability of some groups to conclude that important therapeutic observations were due to antisense mechanisms. This has underlined the need for exhaustive and expensive control ODNs to be used in experiments. Inside cells it is not possible to improve specificity by raising the temperature or changing the ionic strength, strategies which are commonly used *in vitro* to minimise non-specific binding of ASODNs. One approach to improve specificity has been to deploy multiple antisense compounds, each directed against a different site in the same target RNA, and thereby achieve complete target inhibition by 'triangulation'. In addition, efforts have been made to exploit the fact that not all portions of an RNA molecule are equally exposed. For example, if a target sequence occurs in an accessible region of the target mRNA but in a protected region of a bystander, the target will be preferentially destroyed by RNase H. Thus identification of antisense molecules complementary to vulnerable regions on the target mRNA may be a way of improving specificity. However, the complexity of RNA molecules makes this a difficult prospect.

The results of this study illustrate the great potential for TF inhibition by ASODNs. Inhibitions of as much as 43% were seen in the presence of 1μM naked AS1, and in the region of 30-45% in the presence of AS1, AS2 ASJM in the presence of CQ were observed. However, the lack of dose dependency and the fact the control ODNs also caused a degree of inhibition suggests that these inhibitions were not entirely sequence specific. Furthermore, the observation that some of the ODNs tested actually induced TF, suggests that an equilibrium between TF inhibition and induction may have been occurring, possibly explaining the lack of dose dependency. It may also have been possible that the doses of ODN chosen were at the top of the dose response curve. Perhaps an exploration of lower ODN doses might have yielded more promising and

specific results. It should also be noted that during the course of this study, although there was no evidence of an overall high responder phenomenon, a few individuals were seen to possess a population of monocytes 'super positive' for TF on stimulation with LPS (Table 6.2). In these individuals, this population of monocytes was most readily inhibited by ASODN. This raises the question of whether it is therapeutically beneficial to simply reduce the number of cells expressing high TF antigen, thus increasing the number of intermediately expressing cells. Bearing in mind that TF is the cellular receptor for factor VIIa, it could be envisaged that those cells highly positive for TF would bind factor VIIa more readily making them more thrombogenic. To date there have been no reports indicating that monocytes having a higher TF antigen concentration are more thrombophilic than those with more intermediate levels. If this were the case, reduction of TF on these cells in particular by ASODNs would be of great therapeutic benefit.

It is obvious that monocytes are a useful target cell for ASODN therapy, and the potential for inhibiting inducible TF is great. The results of this study suggest that inhibition of TF is possible, but much work needs to be done in designing a more specific, accessible and efficacious ODN to the TF mRNA.

CHAPTER 7

TISSUE FACTOR INDUCTION ON MONOCYTES: POTENCIES OF A PANEL OF DIFFERENT LIPOPOLYSACCHARIDE MOLECULES

7.1 Introduction

Studies of LPS inductions of clotting activity most often utilize *E. coli* LPS. It is clear, however, that LPSs are a complex set of molecular species the activities and functionality of which vary greatly and are dependent on their structures and bacterial source.

7.1.1 Bacterial Lipopolysaccharides

Bacterial lipopolysaccharides (LPS) are complex glycolipids that are integral components of the outer membrane of all Gram-negative bacteria and which induce a series of acute pathophysiological reactions in higher organisms (Table 7.1). However, in addition to toxic effects, LPSs also induce effects considered to be beneficial (Table 7.1).

Biological Effects of Bacterial LPS and/or Free Lipid A	
Lethal toxicity	Activation of complement
Pyrogenicity (Humans, Rabbits)	Activation of procoagulant activity (tissue factor)
Hypothermia (Mice)	Activation of macrophages
Local Shwartzman reaction	Induction of tumour necrosis factor
Limulus amoebocyte lysate gelation	Induction of interleukin-1
Induction of endotoxin intolerance	Induction of interferon
Adjuvant activity	Induction of nonspecific resistance to infection
Induction of colony -stimulating factor	Protection against irradiation
Induction of prostaglandins	Mitogenicity of B lymphocytes
Activation of granulocytes	

TABLE 7.1. Biological Effects of Bacterial LPSs. (Taken from Lindberg *et al.*, 1990)

Structurally, most types of LPS are composed of three distinct regions: the lipid A hydrophobic, the core oligosaccharide (composed of the structurally distinct inner 'lipid A proximal' region which consists of 3-deoxy-D-manno-2-octulosonic acid (2-

keto-3-deoxyoctonate; Kdo) and L-glycero-D-manno-heptose residues, and the outer core which consists of D-glucose and/or D-galactose and *N*-acetyl-D-glucoseamine regions) and the O-polysaccharide which consists of long chains of repeating oligosaccharide units (Fig 7.1). This general structure is typical of the LPS of *Escherichia coli* and related enterobacteria and is often referred to as the smooth or S-form chemotype. Enterobacterial mutants which have lost the O-polysaccharide region of their LPS molecule are described as rough and produce a rough or R-form chemotype in which the core oligosaccharide is the terminal region. However, many Gram-negative species naturally produce a rough form of LPS which is often termed the lipooligosaccharide (LOS). R-mutants of *Salmonella* species and *E. coli* which produce only partial core oligosaccharide are termed deep-rough mutants (Fig 7.2). Such partial structures exist in nature, for example the full LOS/LPS of *Chlamydia* species consists of lipid A and a 3-deoxy-D-manno-2-octulosonic acid (Kdo) oligosaccharide only, which is similar to the deep-rough structure of the Re chemotype - with which it cross reacts serologically (Brade *et al.*, 1987; Holst *et al.*, 1993).

7.1.2 Structural and Antigenic Heterogenicity

The O-polysaccharide structure of S-form LPS determines the O-serotype of the strain and in *E. coli* alone there are over 160 different known serotypes. The core oligosacharide can be structurally and serologically divided into two regions, the inner and outer core. In *E.coli* there are five recognised core structures which show much similarity to each other and to that of *Salmonella* species. The outer cores consist of five hexoses (D-glucose, D-galactose and D-glucosamine) in different arrangements and configurations, but showing an overall degree of similarity (Fig 7.1). The inner part of the core is much more conserved containing the Kdo and heptose trisaccharides as well as phosphate substituents. There is little variation between closely related species. The lipid A region is much more conserved and within a species or group of closely related species may be identical or only differ in minor ways.

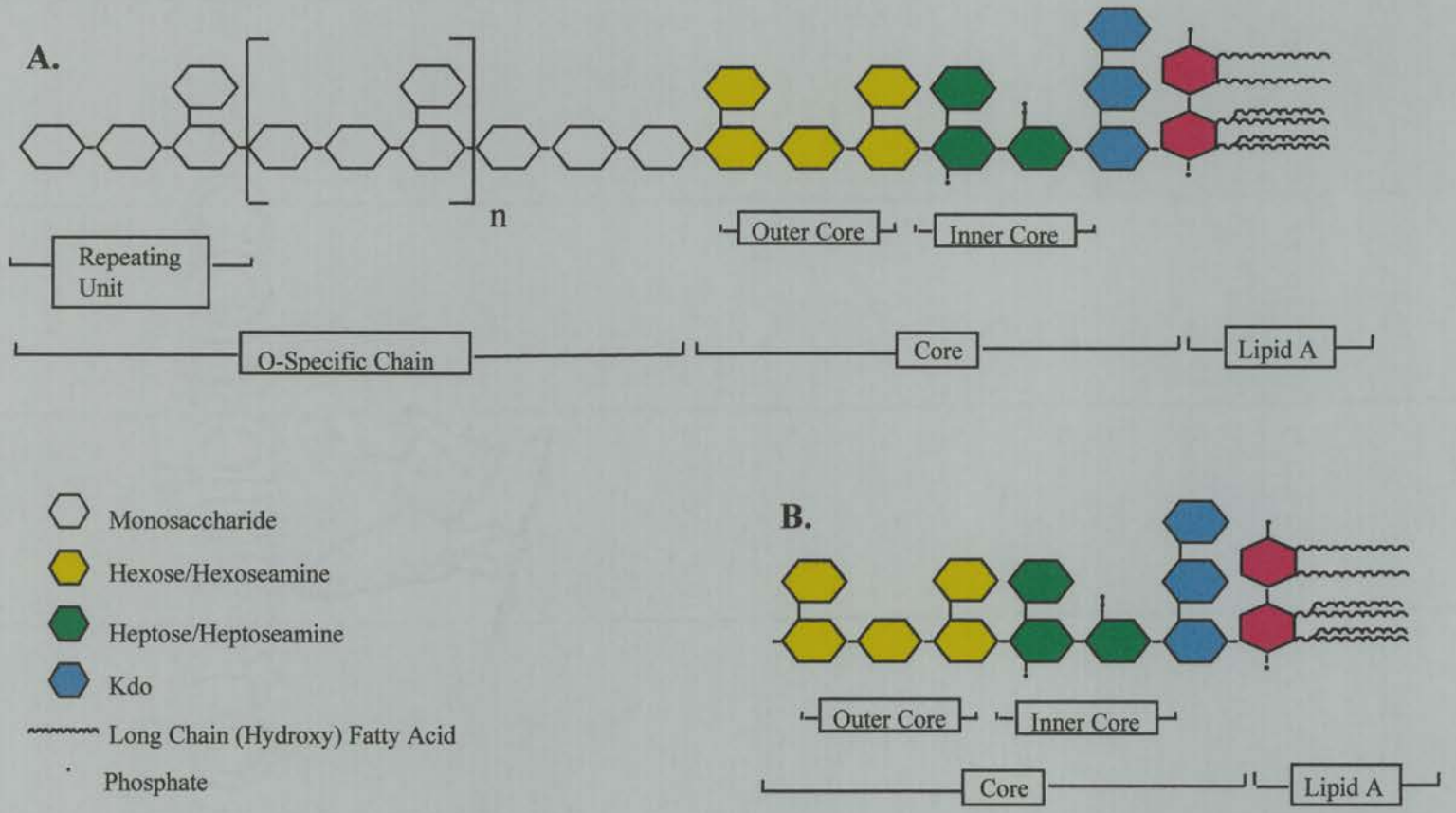


FIGURE 7.1 Schematic Representation of **A.** Smooth and **B.** Rough LPS. Adapted from Lindberg *et al.*, 1990.

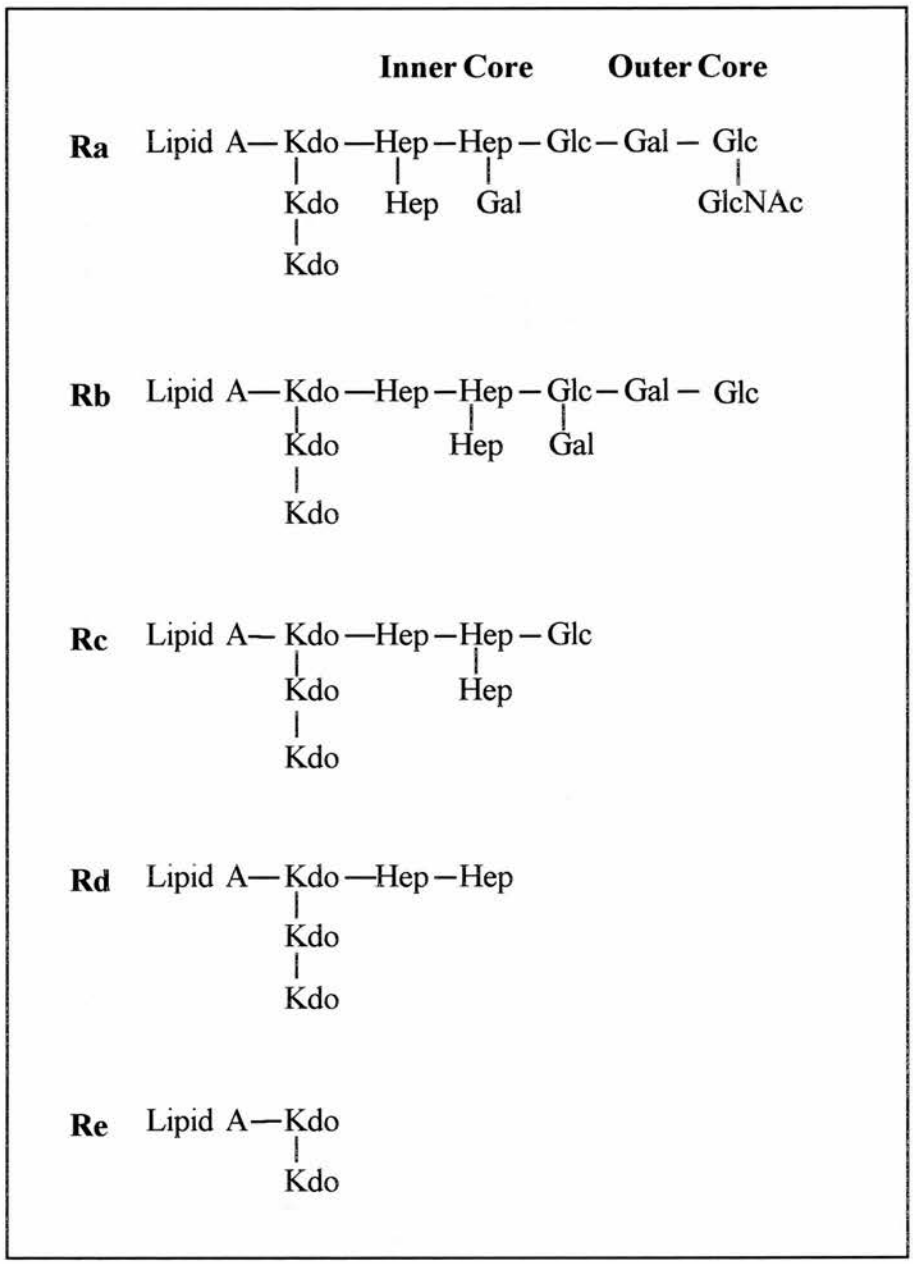


FIGURE 7.2 Chemical structures of *Salmonella* R-mutants. Kdo=3-deoxy-D-*manno*-2-octolosonic acid, Hep=heptose, Glc= D-glucose, Gal=D-galactose, GlcNAc=*N*-acetyl-D-glucosamine. Adapted from Poxton, 1995.

7.1.3 Function of LPS

The minimal structure of the LPS molecule which is essential for the viability of the bacterium is equivalent to the Re-form LPS (Rietschel *et al.*, 1990). The lipid A of LPS is the sole lipid found in the outer leaflet of the bilayer of the outer membrane, and the number and arrangement of the substituent fatty acids results in a membrane of extremely low fluidity. It functions as a permeability barrier to hydrophobic molecules and is, therefore, structurally and physiologically important The long O-polysaccharide chains in the pathogenic bacterium confer resistance to the lytic action of serum complement by preventing the insertion of an active membrane attack complex into the bacterial envelope. Most R-mutants or naturally rough bacteria are thus sensitive to the lytic action of normal serum.

7.1.4 Endotoxin

Endotoxin is the name given to bacterial LPS which is present within the circulation. The lipid A region is responsible for the endotoxicity of the molecule but is never found separated from the Kdo, the Re-like structure being the smallest found naturally. Thus the minimum structure needed for endotoxicity is similar to that required for bacterial survival. The host is continuously being exposed to endotoxin and as a result, several mechanisms have evolved for detoxifying large and small amounts of LPS. For example, on leukocytes, there are three classes of LPS receptors. CD18 binds to LPS present on bacteria and promotes their phagocytosis without stimulating any secretory reponse (Wright and Jong 1986). Scavenger receptor (acetyl-low density lipoprotein) recognises dispersions of LPS and is involved in pinocytosis and subsequent degradation of LPS. Finally, LPS-binding protein (LPB), an acute phase protein (Wright, 1991) which is thought to be involved in the neutralization of LPS, mediates the secretory responses of leukocytes through binding to CD14 (Wurfel *et al.*, 1994). The LPS/LPB complexes act primarily on monocytes/macrophages through the CD14 receptor inducing the secretion of TF in addition to a range of proinflammatory cytokines.

7.1.5 *Bacteroides fragilis* LPS

The gram-negative bacterium *Bacteroides fragilis* is the most commonly isolated anaerobic species in many infectious processes arising from faecal contamination such as intraabdominal absesses and peritonitis (Gorbach and Bartlett 1974). Anaerobes in general constitute approximately 99% of the faecal bacterial mass and, of this amount, *Bacteroides* species constitute 20-30% of species isolated (Duerden *et al.*, 1991). *Bacteroides* species are numerically more abundant in the gut outnumbering members of the family Enterobacteriaceae such as *Escherichia coli* by about 1000 fold (Delahooke *et al.*, 1995) and as such they constitute a large pool of biologically active LPS. *Bacteroides fragilis* has been the most extensively studied of the *Bacteroides* species (Lindberg *et al.*, 1990; Williamson *et al.*, 1984). It has been well documented that *Bacteroides fragilis* LPS is generally less endotoxic than enterobacterial LPS (100-1000 fold less) has a different fatty acid structure and is monophosphorylated (Lindberg *et al.*, 1990). Early studies on the endotoxic activity of the LPS of *Bacteroides fragilis* were done in the 1970s by Sveen *et al.*, 1977, Sveen, 1997 and Kasper *et al.*, 1976. It was concluded that the endotoxic activity of *B. fragilis* was lower than that seen with the LPS of *Salmonella*. More recently, the biological activity of *B. fragilis* LPS has been reassessed by examining the lipid A molecule. It has become apparent that the phosphate groups on the glucosamine residues are important as are the number and structure of the fatty acids linked to the amino sugars (Fig 7.1), (Kotani *et al.*, 1985; Rietschel *et al.*, 1987a; Rietschel *et al.*, 1987b). Loppnow *et al.*, 1986, have shown that absence of the phosphate group on the non-reducing glucosamine of the LPS (substance 505) reduced pyrogenicity in rabbits by 100-fold compared with its phosphorylated *E. coli*-like derivative (substance 506). In addition, the pyrogenic activity in rabbits of a fully phosphorylated substance with four instead of six fatty acids (substance 406) was reduced by >10-fold and the substance had lost its ability to induce interleukin 1 in monocyte cultures. Moreover, futher studies by Loppnow *et al.*, 1988 in which a characterised LPS of *B. fragilis*, NCTC 9343, was used demonstrated that, compared with the LPS of *Salmonella minnesota* Re595, its ability to induce interleukin 1 was reduced by 100- to 1000 fold.

In addition, the ability of the LPS of *B. fragilis* to stimulate a release of prostaglandins PGE_2 and $PGF_{2\alpha}$ was slightly reduced from that of the LPS from *S. minnesota* Re595. The LPS of *B. fragilis* NCTC 9343 lacks the phosphate group on the non-reducing glucosamine and has an average of 5 branched and long chain (C15-C17) fatty acids (Weintraub *et al.*, 1989) and so this has been concluded to be the cause of the low endotoxicity.

However, recent studies by Delahooke *et al.*, 1995 have shown that the biological activity of *B. fragilis* LPS is dependent on the extraction method. The phenol-chloroform-petroleum method used in earlier studies results in an LPS of low endotoxic activity. However the aqueous phenol method used by Delahooke *et al.*, produces a product with biological activities equivalent to that of enterobacterial LPS. It is *Bacteroides fragilis* LPS extracted by the aqueous phenol method that is used in the current study.

7.2 Materials and Methods

7.2.1 LPS Molecules Tested

Four of the LPS molecules tested were isolated and extracted by Prof. Ian Poxton, Department of Medical Microbiology, University of Edinburgh and were kindly donated in the lyophilised form. The *Escherichia coli* O18K$^-$ LPS and the *Bacteroides fragilis* LPSs were extracted using the aqueous phenol method (Westphal and Luderitz 1954) as described by Hancock and Poxton, 1988 and the *Salmonella minnesota* Re595 and Ra60 strains were isolated using the phenol-chloroform-petroleum method (Galanos *et al.*, 1969). All native LPSs were made free from protein contamination by treatment with proteinase K (20μg/ml) at 65°C for 2 hours. Proteinase K was removed by two washes with pyrogen free water at 100,000 x g. *Escherichia coli* O111:B4 LPS was extracted by the trichloroacetic acid method (Staub *et al.*, 1965) and was purchased from Sigma. This latter LPS contains more protein than the other LPSs and so will be less pure (Poxton, personal communication).

7.2.2 TF Inducing Ability of a Panel of Different LPS Molecules

Whole blood samples from a total of six healthy volunteers were collected into citrate coated tubes (Sarstedt). Aliquots of 500μl were placed in sterile polypropylene tubes and were treated with a range of concentrations of the 5 different LPS molecules (reconstituted in sterile, pyrogen free distilled water) to be tested (0.1pg/ml-10μg/ml) (see table 7.1). The blood samples were incubated for 2 hours at 37°C in an atmosphere of 95% air 5% CO_2. After this time, 100μl aliquots of blood were indirectly immunostained for tissue factor as described in chapter 2, and the fixed cells subsequently analysed by flow cytometry. The percentage TF positive monocytes was recorded for each dose of each of the LPS molecules tested and dose response curves plotted. From these curves the ED_{30} value was calculated. This is defined as the dose of LPS required to produce 30% positive monocytes.

7.2.3 Incubation of *E. coli* O18K$^-$ LPS with an Excess of *Bacteroides fragilis* LPS

To test the hypothesis that *B. fragilis* LPS masks the TF inducing ability of *E. coli* O18K$^-$ LPS, 500µl aliquots of blood were incubated with a range of concentrations of *B. fragilis* LPS (0.1pg/ml-10µg/ml) either in the presence of a 1ng/ml dose of *E. coli* O18K$^-$ LPS or sterile distilled water for 2hrs at 37°C 95% air, 5% CO_2. Aliquots (100µl) of blood were then indirectly immunostained for tissue factor and analysed by flow cytometry. The percentage TF positive monocytes was recorded both in the presence and in the absence of the *E. coli* O18K$^-$ LPS.

Bacterium	Strain	Description of LPS/Strain
Escherichia coli	O111:B4	Standard smooth LPS purchased from Sigma
Escherichia coli	O18K$^-$	LPS from commonest O type in septicaemia
Salmonella minnesota	Ra60	Full core rough LPS from an Ra mutant strain
Salmonella minnesota	Re595	Deep rough LPS from an Re mutant strain
Bacteroides fragilis	NCTC9343	Smooth LPS from one of the commonest inhabitants of the gut

TABLE 7.2 Description of LPS Molecules Tested

7.3 Results

7.3.1 Potencies of a Panel of LPS Molecules at Inducing MonocyteTissue Factor Antigen

Whole blood was incubated with increasing doses of 5 different LPS molecules and the dose of LPS required to produce 30% positive monocytes was calculated (ED_{30}). The results revealed the following increasing order of LPS potencies: *B.fragilis* was the least potent at inducing TF (ED_{30} 300ng/ml). The LPS from *E. coli* strain O111:B4 was more potent (ED_{30} 0.2 ng/ml). The LPS from *E. coli* O18K⁻ strain and the *S. minnesota* strain Ra60 were of similar potency (ED_{30} 0.07ng/ml) and the smallest deep rough LPS Re595 from *S. minnesota* was slightly more potent (ED_{30} 0.03ng/ml) (Table 7.3 and Fig. 7.3)

LPS	Approximate ED_{30} For TF Induction (ng/ml)
E. coli O111:B4	0.2
E. coli O18K	0.07
S. minnesota Ra60	0.07
S. minnesota Re595	0.03
B. fragilis	300

TABLE 7.3 Potencies of LPS Molecules Tested at Inducing TF Antigen on Monocytes in Whole Blood. ED_{30} is defined at the dose of LPS required to produce 30% positive monocytes.

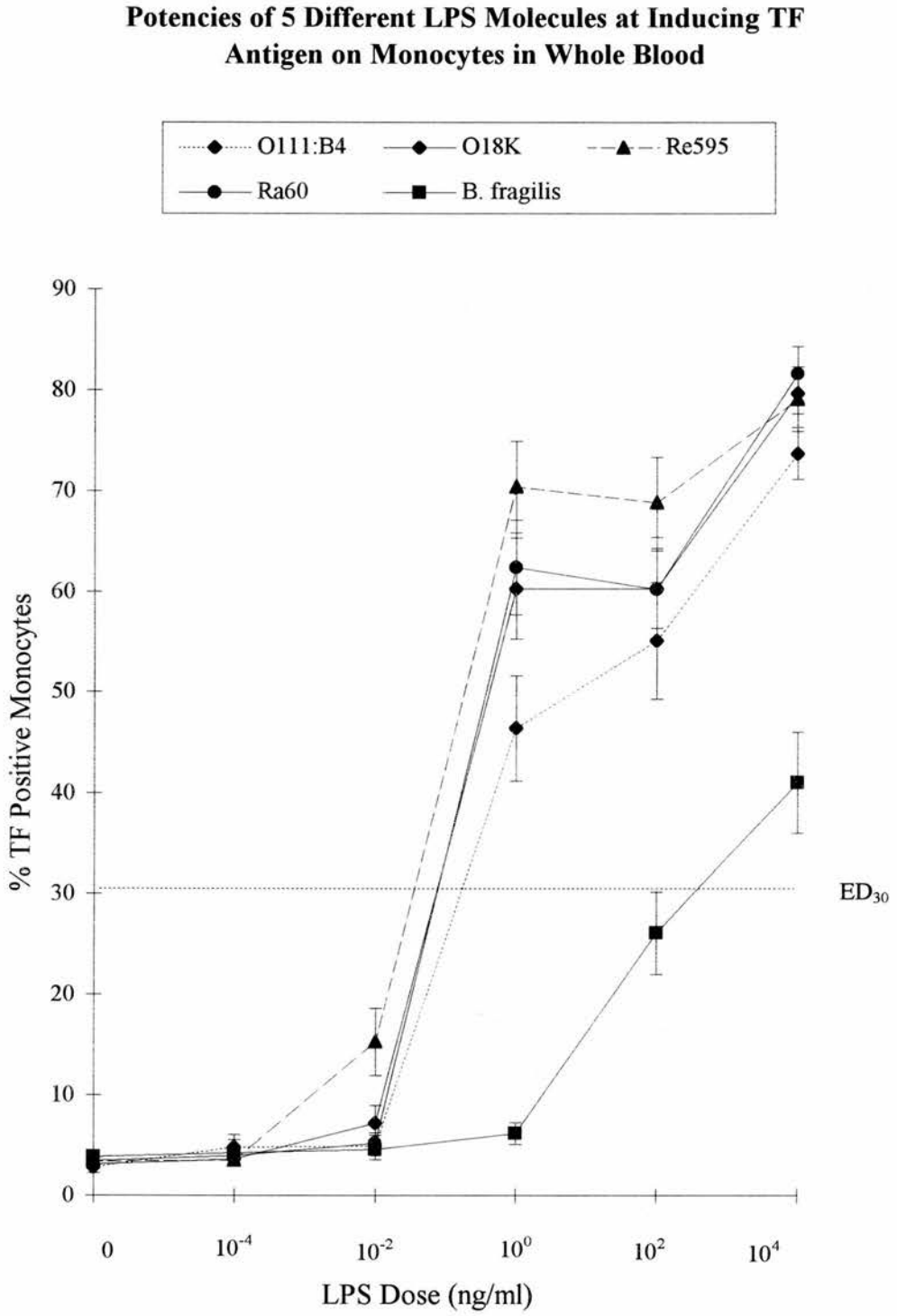


FIGURE 7.3 Potencies of 5 Different LPS Molecules at Inducing TF Antigen on Monocytes in Whole Blood. It can be seen that *B. fragilis* LPS is the least potent (ED_{30} 300ng/ml). Salmonella minnesota Re595 LPS was the most potent (ED_{30} 30pg/ml). ED_{30} is defined as the dose of LPS required to produce 30% positive monocytes. Points are the means of 6 separate experiments +/- sem.

7.3.2 Effect of *B. fragilis* on TF Induction by *E. coli* O18K⁻ LPS

A set dose of 1ng/ml *E. coli* O18K LPS was added to whole blood and incubated in the presence of increasing concentrations of *B. fragilis* LPS. When added in excess, *B. fragilis* LPS was able to mask the TF inducing ability of *E. coli* O18K⁻ LPS (Fig 7.4). At a dose of 1 ng/ml, *E. coli* O18K⁻ LPS produced 70% TF positive monocytes which was gradually reduced by a maximum of 65% to 45% TF positive monocytes on addition of increasing concentrations of *B. fragilis* LPS (0.1pg/ml-10μg/ml) (Fig 7.4).

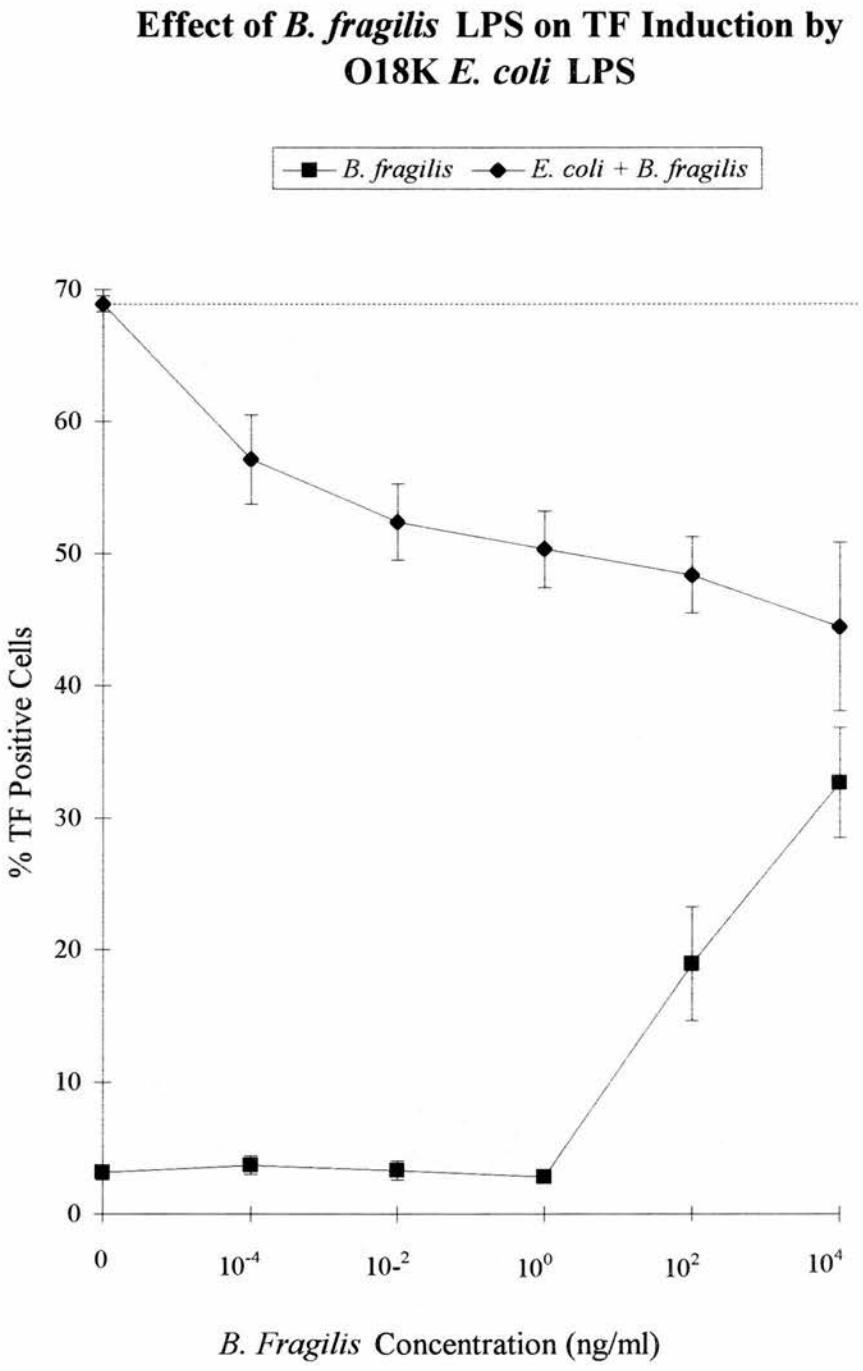


FIGURE 7.4 Effect of *B. fragilis* LPS on TF induction by *E. coli* O18K⁻ LPS. Increasing concentrations of *B. fragilis* LPS were added to whole blood containing a set dose of 1ng/ml *E. coli* O18K⁻ LPS. The presence of an excess of *B. fragilis* LPS resulted in a 65% reduction in the TF induction by *E. coli* O18K⁻ LPS. Points are the mean of 6 separate experiment +/- sem.

7.4 Discussion

Bacterial LPSs or endotoxins are vital components of the outer cell walls of Gram-negative bacteria. A variety of pathophysiological responses in various tissues and organ systems occur during endotoxaemia including the induction of procoagulant proteins such as TF and proinflammatory cytokines such tumour necrosis factor alpha (TNF-α) on peripheral blood monocytes. Over stimulation of the clotting and immune systems by excessive LPS leads to symptoms of endotoxic shock or systemic inflammatory response syndrome (SIRS) a condition characterized by disseminated intravascular coagulation and multiple organ failure (Bone *et al.*, 1993). Therefore, bacterial LPSs have been a focus of research for several decades.

The purpose of this preliminary study was to investigate the TF inducing capability of a range of different bacterial LPS molecules. The five different molecules chosen were: *E. coli* O111:B4 LPS, which is a standard LPS used in many studies requiring induction of TF (including Stephens and Rivers, 1996, Stephens *et al.*, 1996, Amirkosravi *et al.*, 1996, Yang *et al.*, 1995, Steinemann *et al.*, 1994); *E. coli* O18K$^-$ LPS which is the commonest O-type found in septicaemia; two *Salmonella* mutant LPSs *S. minnesota* Ra60 LPS which is a full core rough LPS and *S. minnesota* Re595 LPS a deep rough LPS; and finally *B. fragilis* LPS which is a smooth LPS from one of the commonest inhabitants of the gut.

It has been concluded that the endotoxic activity of *B. fragilis* is lower than that seen with the LPS of *Salmonella* (Sven *et al.*, 1977; Kasper *et al.*, 1976). Recently, the apparent reduced biological activity of *B. fragilis* LPS has been associated with the structure of the lipid A molecule. It has become apparent that the phosphate groups on the glucosamine residues are important as are the number and structure of the fatty acids linked to the amino sugars (Kotani *et al.*, 1985; Rietschel *et al.*, 1987a; Rietschel *et al.*, 1987b). It has been shown that LPS from *B. fragilis* NCTC 9343 is less potent at inducing IL-1 in monocyte cell cultures than LPS from *S. minnesota* Re595 and at stimulating the release of prostaglandins PGE_2 and $PGF_{2\alpha}$ (Loppnow *et al.*, 1988). The LPS of *B. fragilis* NCTC 9343 lacks the phosphate group on the non-

reducing glucosamine and has an average of 5 branched and long chain (C15-C17) fatty acids and so this has been concluded to be the cause of the low endotoxicity (Weintraub *et al.*, 1989).

Studies by Delahooke *et al.*, 1995 have demonstrated that the stimulation of TNF-α production by *Bacteroides fragilis* LPS in isolated human mononuclear cells is between 2 and 4 fold less than that by *E. coli* O18K⁻ LPS. This group have also shown that this induction of TNF-α is through a CD14 independent mechanism, as it is not blocked by anti-CD14 monoclonal antibodies. In the current study I have shown that *B. fragilis* LPS is 1500 times less potent at inducing TF antigen on monocytes than *E. coli* O111:B4 (ED_{30} 200pg/ml vs 300ng/ml), 3000 times less potent than *E. coli* O18K⁻ LPS or *S. minnesota* Ra60 LPS (ED_{30} 100pg/ml vs 300ng/ml), and 10,000 times less potent than *S. minnesota* Re595 LPS (ED_{30} 30pg/ml vs 300ng/ml) where ED_{30} is defined as the dose of LPS required to produce 30% TF positive monocytes. To my knowledge, there are no other studies comparing the potencies of different LPSs at inducing TF antigen on monocytes. It is possible that the extraction methods used to produce the LPS molecules might effect their TF inducing ability. However, this is unlikely as the least potent *B. fragilis* LPS was extracted using the aqeous phenol method, which has been shown by Delahooke *et al.*, to produce LPS of a high biological activity, in contrast to the more potent Re595 LPS and Ra60 LPS which were extracted using the less effective phenol-chloroform-petroleum method. The diminished potency of *B. fragilis* LPS at inducing TF compared with the others tested is much more pronounced in my study than that seen by Delahooke *et al.*, for TNF-α production. This may be due the the fact my studies were carried out in a whole blood model rather than in isolated cells. Although anti-TNF-α antibodies have not been shown to attenuate procoagulant responses in experimental and clinical experiments (Salat et al., 1996) TNF-α has been shown to enhance LPS induction of TF in monocytes and endothelial cells (Parry and Mackman, 1994). *In vitro* studies have shown the tissue factor gene to be under the control of a *cis*-acting NFκB site (Oeth *et al.*, 1994; Parry and Mackman 1994) and TNF-α directly contributes to activation of this transcription factor leading to upregulation of TF expression (Parry and Mackman 1994). It is possible that the

diminished induction of TNF-α by *B. fragilis* coupled with the reduced capacity of *B. fragilis* to directly induce the TF protein resulted in the significantly lower TF antigen expression on monocytes seen with *B. fragilis* LPS in my study. In a recent study by Lynn *et al.*, 1993, CD14 and serum were shown not to be absolutely necessary for the activation of mononuclear phagocytes by bacterial LPS. They suggested that a CD14 independent pathway may be of importance in local sites of infection where the concentration of LPS may be high, such as in faecal contamination of the peritineum. Delahooke *et al.*, have shown that in peritoneal macrophages from C3H/HeJ (LPS non-responders) and C3H/He N (LPS responders) mice TNF release after stimulation with both *B. fragilis* and *E. coli* LPS appeared to occur through a CD14-independent pathway. The current study does not provide any evidence that *B. fragilis* LPS is acting via a CD14 independent pathway. Studies using CD14 neutralizing antibodies would be required to confirm this.

Studies by Loppnow *et al.*, in monocytes isolated from rabbits have demonstrated that, compared with the LPS of *Salmonella minnesota* Re595, the ability of *B. fragilis* LPS to induce interleukin 1 was reduced by 100- to 1000 fold (Lopponow *et al.*, 1988). Similarly, in the current study, I have demonstrated that *B. fragilis* LPS is 1500 times less potent than *S. minnesota* Re595 LPS at inducing TF antigen on monocytes. The deep rough structure of *S. minnesota* Re595 is very similar to the structure of *Chlamydial* LPS, which has been implicated in the pathogenesis of coronary heart disease and in the aggravation of atherosclerosis (Leinonen *et al.*, 1993). The results from the current study show that, of all the LPSs tested, *S. minnesota* Re595 LPS was the most potent at inducing TF on monocytes in their whole blood environment. It is possible that this increased ability to induce TF may contribute to the pathophysiology of *Chlamydia* induced cardiovascular disease.

It has been shown by Delahooke *et al.*, that an excess of *Bacteriodes* LPS blocked the effects of *E. coli* LPS on human mononuclear leuckocytes and THP-1 (monocytic) cells. Whether this was due only to the configuration of LPS in solution or due to an effect at the cellular level was not known. There is a well documented synergistic relationship between *E. coli* and *B. fragilis*, whereby *B. fragilis* inhibits the phagocytic killing of *E. coli* (Onderdonk *et al.*, 1976; Rotstein *et al.*, 1989).

Furthermore, Magnuson *et al.*, have shown that *B. fragilis* NTCT 9343 LPS inhibits *E. coli* LPS-induced human endothelial cell adhesiveness for neutrophils (Magnuson *et al.*, 1989). In the current study I have shown that *B. fragilis* LPS inhibited the induction of TF antigen on monocytes by *E. coli* O18K$^-$ LPS by 65%. It is known that *Bacteroides* species outnumber *E. coli* in the gut by at least 1000-fold. The results of the present study, taken with the observations from the studies mentioned suggest that if *Bacteroides* LPS is the main inducer of TF in situations where bacteria or their products translocate from the gut into the blood stream, *B. fragilis* should be targeted rather than *E. coli*. It is not yet known if the observations seen *in vitro* will occur *in vivo*. However, if this is the case, it is not unlikely that the *Bacteroides* LPS may serve a protective role against *E. coli* LPS induced monocyte TF expression.

Infection with *Chlamydia pneumoniae* has recently been shown to be associated with atherosclerosis. Fryer et al., 1997 have shown that *Chlamydia pneumoniae* can infect cultured human endothelial cells and stimulate a four-fold increase in the expression of TF and platelet adhesion thus providing a link between infection with *C. pneumoniae* and procoagulant activity. In addition, the *C. pneumoniae* organism has been detected in atherosclerotic plaques from coronary arteries (Kuo et al., 1995), carotid arteries and the thoracic and abdominal aorta (Ong et al., 1996). In the current study I have shown that of all the LPSs tested, *S. minnesota* Re595 LPS was the most potent at inducing TF expression on monocytes. Bearing in mind that the structure of *Chlamydia* LPS is similar to that of *S. minnesota* Re595 LPS (they cross react serologically) (Brade et al., 1987; Holst et al., 1993) it is possible that in patients infected by *C. pneumonia*, *Chlamydia* LPS may be responsible for the increased thrombogenicity of the atheroscerotic plaque.

CHAPTER 8

GENERAL DISCUSSION AND FUTURE WORK

8.1 General Discussion and Future Work

The main aim of this study was to investigate the potential of the monocyte as a therapeutic target for the inhibition of the procoagulant protein tissue factor. Tissue factor is the major *in vivo* activator of blood coagulation leading to thrombin generation and fibrin deposition. Although TF is constitutively expressed in many extravascular cells, peripheral blood cells do not normally express TF. Monocytes and endothelial cells can be induced to express TF in response to a variety of agonists such as LPS and TNF- α and, as a result, play an important role in the pathophysiology of coagulation disorders such as disseminated intravascular coagulation.

Antisense oligonucleotides are short lengths of single stranded DNA which bind to DNA or mRNA and prevent the trascription or translation of target proteins. As such, antisense therapy provides the promise of specific inhibition of particular target proteins. The experiments described in chapter 3 investigate the potential of the monocyte as a target for antisense therapeutics, with the view to specific inhibition of the TF protein. Using the 25mer phosphorothioate ASODN GEM-91 I have shown that there is heterogeneous uptake of ODN between PBL subtypes with monocytes in particular internalizing ODNs more efficiently than other cell types. In addition, I have demonstrated that uptake is dose and time dependent. After 4 hours in culture with 10μM ASODN, monocytes internalized approximately 45 times more ODN than lymphocytes and twice as much as neutrophils, and at this dose uptake was not yet saturated. Indeed at this dose monocytes contained 828fg ODN/cell which equates to $5x10^7$ molecules of single stranded DNA. It is not known exactly how many copies of ASODN are required to produce complete inhibition of target mRNAs but this amount is likely to be in excess. However, from this study the intracellular fate of the ODN was not determined. It has been noted that ASODNs have their effects mostly in the cytoplasm where they come into contact with their target mRNA (Wagner *et al.*, 1995). Although I have shown that monocytes internalize ODN more efficiently

than other PBL subtypes, it is unclear as to whether the ODN reaches the cytoplasm and its target mRNA. Studies have been carried out which conclude that ASODN uptake is, at least in part, receptor mediated, whereby ODNs bind to a cell surface receptor and are internalized into 'receptosomes' from where they may or may not escape into the cytoplasm (Beltinger *et al.*, 1995). Other groups have demonstrated that ODNs must be conjugated with a carrier molecule or encased in a liposome to facilitate entry into cells and improve intracellular bioavailability (Hartmann *et al.*, 1998). These carriers have been shown to be cytotoxic at certain concentrations and so may not be appropriate for systemic administration of ODNs. In agreement with Hartmann et al, I have shown that monocytes internalize more ODN than other PBL subtypes. However, in my study the differences are more striking, suggesting that sufficient monocyte loading may be achieved at lower doses than previously thought and without the need for carrier molecules. This suggests that monocytes are potentially good target cells for antisense therapy. It was not possible in the current study to examine the intracellular fate of ASODNs within the monocyte. Using confocal and electron microscopic techniques, it has been suggested by some groups that ODNs localize to intracellular acid vesicles (Loke et al., 1989; Stein et al., 1993; Bennett et al., 1992) whilst others have observed ODNs in the nucleus and free in the cytoplasm (Beltinger et al., 1995). Further microscopic studies will be required to fully investigate the intracellular distribution of ASODNs in monocytes and to track the movement of ODNs from the cell surface to the target mRNA.

Having shown that monocytes internalize more ODN than other PBL subtypes, an investigation into a possible mechanism for this was investigated. The pattern of uptake of ODNs into monocytes was strongly suggestive of a receptor mediated mechanism. It has been demonstrated by many groups that ODNs enter cells by a receptor mediated mechanism and a number of candidate receptors have been investigated. Benimetskya *et al.*, 1997 have convincingly demonstrated that the Mac-1 (CD11b/CD18) receptor binds ODNs in neutrophils. This receptor is found in greatest abundance on monocytes and neutrophils and is a likely candidate for ODN uptake in monocytes. In addition it was thought that the monocyte cell surface

marker CD14 may also be affected by ODN molecules In chapter 4, the effect of ODN on monocyte cell surface Mac-1 and CD14 was investigated. It was shown that fluorescently labelled ODN association was greatest in the cells which had highest cell surface Mac-1 and CD14 receptor levels, namely monocytes and neutrophils. Furthermore, it was demonstrated that incubation of PBLs with ODN for 30 minutes resulted in a significant decrease in the monocyte cell surface levels of Mac-1 which decreased further over the 4 hour incubation period. This mirrored the rate of internalization of ODN described in chapter 3. CD14 levels also significantly decreased, but not until after 4 hours in the presence of GEM-91. Although it is clear from these studies that the ODN was causing a downregulation of Mac-1 and CD14, it is not clear what form this downregulation is taking. It is possible that the ODNs were simply binding to Mac-1 and CD14 preventing antibody recognition of the receptors by flow cytometry. However, the uptake kinetics of ODN described in chapter 3, suggest that once bound to the cell surface, ODNs are indeed internalized. This possible receptor mediated endocytosis would be responsible for a decrease in cell surface receptors. Alternatively, the ODN may be causing a change in monocyte cell phenotype by signalling a downregulation in receptor number. Both the Mac-1 and CD14 receptors are involved in the LPS induction of TF on monocytes, and so any effect of ODNs on these receptors could contribute to any modulation of ASODNs on TF expression If ODNs are indeed ligands for these monocyte receptors, it is possible that they could initiate intracellular signalling pathways resulting in the expression of TF or other inducible proteins. Benimetska *et al.*, have shown that upregulation of cell surface Mac-1 by TNF-α increased the binding of ODNs. During the course of this study I attempted to demonstrate an increased ODN association with monocytes after upregulation of Mac-1. However, this proved to be outwith the limits of my experimental design. Although an upregulation in Mac-1 was demonstrable with TNF-α, further treatment of isolated PBLs with ODN resulted in a loss of monocyte morphology, and so flow cytometric analysis was not possible. To further clarify the role of Mac-1 in ODN uptake in peripheral blood monocytes, experiments using Mac-1 neutralizing antibodies are required. By examining ODN uptake in the presence of a Mac-1 neutralizing antibody, or in the presence of a

known ligand for Mac-1 such as fibrinogen a more conclusive effect of the importance of this receptor in ODN uptake could be determined.

Having determined that monocytes effectively internalized ODN, it was concluded that it may be possible to inhibit induced TF expression on monocytes by ASODNs to TF. The observations of over a decade of studies by Østerud et al, have concluded that TF induction in monocytes is strongly influenced by cellular cooperations between monocytes, neutrophils and platelets in whole blood. Therefore, I decided that monocytes should be studied in the context of *ex vivo* whole blood in an attempt to provide as physiological a model as possible in which to investigate TF induction and its regulation. To this end it was necessary to characterize the induction of TF by LPS in monocytes in *ex vivo* whole blood to determine the dose and time of incubation of LPS which provided a suitable and reproducible TF induction. It was decided to combine the methods of Østerud and Amirkhosravi *et al.*, to produce a suitable system. The final protocol involved the incubation of citrated whole blood with 10μg/ml LPS for 2 hours, after which time it immunostained for TF and analysed by flow cytometry. During the course of this study it was shown that, in agreement with the work of Østerud and Amirkoshravi, there was little variation in the TF **antigen** inducing ability of different individuals when treated with 10μg/ml LPS. Østerud has, however, noted that TF **activity** varies dramatically between the highest and lowest responding individuals by as much as 50-fold. These results suggest that low TF antigen levels may translate into much higher TF activities, and emphasize the case for therapeutically targeting TF antigen levels by antisense ODNs in preference to activity inhibition with, for example factor VIIai.

In chapter 6 studies aimed at the inhibition of TF induction using specifically designed ASODNs are described. I initially designed a 30 base phosphorothioate ASODN targeted to bind around the start-site of translation of the TF mRNA (ASJM) in an attempt to inhibit translation of the protein, as I believed that was likely to be an accessible site on the mRNA. This ODN alone did not produce any significant inhibition of LPS induced TF antigen. During the course of these experiments a study

by Stephens and Rivers was published in which they reported an 80% inhibition of TF **activity** in **isolated** monocytes with a specifically designed ASODN to TF (AS1). This ODN, in addition to a truncated version of it (AS2), were used in the current study to investigate inhibition of TF antigen. At the 1μM dose a significant (42 %) inhibition was seen in TF antigen when compared with ASODN untreated cells. Neither the truncated ODN nor the mismatched version had an effect. In an attempt to produce specific antisense effects, Stephens and Rivers' ODN was designed to contain the rare tripeptide motif Trp-Lys-Ser, which had been predicted as a functional motif involved in the interaction with serine proteases,. My studies initially demonstrated that AS1 and its truncated and mismatched counterparts actually induced TF on monocytes whereas ASJM and GEM-91 did not. Despite this fact, AS1 was better at inhibiting TF than ASJM. This may have been due to the fact that, through it's possible increased interaction with either the CD14 or Mac-1 receptors, AS1 gained better entry to the cell by receptor mediated endocytosis. The concurrent induction of TF may have been an unfortunate by product of this interaction, which was not picked up by Stephens and Rivers. The increased efficacy of AS1 is unlikely to be wholly a consequence of AS1 superior design, as in the presence of the lysosmatropic agent chloroquine, both AS1 and ASJM inhibited TF antigen induction to roughly the same extent 45 ± 6.7% vs 37 ± 7.7 % and in regime 2 ASJM produced a 41.9 ± 9.01% inhibition. This suggests that the disappointing results seen without CQ were more likely to be due to diminished intracellular bioavailability which was improved in the the presence of the lysosomatropic agent. However, it has been reported that the efficacy of ASODNs is better *in vivo* than *in vitro* (Monia et al.,1996) so it is possible that the effects of these ODNs could be improved if administered systemically *in vivo*. It would be interesting to investigate the potential of systemic administration of ASODNs to TF in an animal model of sepsis, to see how they compare with monoclonal antibodies to tissue factor, which have been shown to decrease mortality in many animal models of DIC (Taylor *et al.*, 1991; Dackiw, 1996). If efficacious, ASODNs to TF would represent a potentially much less toxic and more specific inhibition of TF. ASODNs to TF could also have applications for more localized therapies. Expression of TF has been shown to increase around the

sites of balloon angioplasty (Weiss *et al.*, 1989). Local administration of ASODNs to TF, perhaps by coating the balloon, would offer potential specificity as well as rapid inhibition of induced TF only, around the site of injury.

Although in the current study the control ASODNs chosen were less efficacious than the ASODNs to TF, it was obvious that some inhibition was occurring with these control molecules. Ideally, several controls should be deployed alongside the specific ASODN containing increasing numbers of specific base mismatches. Although a random mismatched ODN was deployed with AS1 and was shown to have a lesser effect (Table 6.4), financial constraints meant that a complete range of control ODNs, for ASJM was not synthesized. In order to conclusively prove that the inhibitory effects seen were indeed true antisense effects, a study using a more sophisticated panel of control ODNs, each control perhaps containing an increasing number of base mismatches, would be required. According to Branch, 1998, the concept that an antisense molecule can selectively knock out a single gene remains, as yet, unconfirmed. The original concept that ODNs are exquisitely specific and easy to design has been marred by the discovery of numerous mechanisms of action leading to non-antisense effects. As discussed already these include binding of the ODN to the target protein preventing its recognition; binding of the ODN to a receptor causing induction of the target protein or indeed another unrelated protein; or binding of the ODN to a non-target RNA ultimately resulting in a decrease of the target protein. Although these non-specific effects may be useful therapeutically, their unpredictability makes them difficult to investigate as the rules for rational drug design cannot be applied, leaving non-antisense effects to be monitored empirically. Although the issue of their ultimate specificity remains, there is growing evidence that antisense molecules can be useful pharmacological tools when applied carefully (Crooke and Bennett, 1996). In addition, certain non-antisense effects of ASODNs promise to be valuable therapeutically. The time and expense necessary to screen large numbers of potential antisense molecules and to monitor their *in vivo* effects, limits their investigation to the pharmaceutical industry. It has been shown in this study that there is merit in targeting the monocyte to inhibit TF induction, but whether or not the inhibitory effects are true antisense effects remains to be seen.

Throughout the course of these studies, *E. coli* O111:B4 LPS has been used to induce TF on monocytes. However, in reality, LPS from a number of different bacteria might challenge the host. *Bacteroides* species are numerically more predominant (by at least 10^3 fold) in the gut than members of the family *Enterobacteriaceae* such as *E. coli*, and as such they represent a potentially large pool of biologically active LPS. It is possible that TF might be induced on monocytes/macrophages by LPS from *Bacteroides fragils* if it were to enter the bloodstream, such as in the case of DIC caused by gut rupture. Furthermore, it has been demonstrated that *B. fragilis* LPS is between 100 - 1000 fold less endotoxic than other LPSs (Lindberg *et al.*, 1990). In the light of studies by Delahooke *et al.*, 1995 which have shown that *B. fragilis* is between 2 and 4 fold less potent at inducing TNF-α in macrophages than *E. coli* LPS, I have investigated the TF antigen inducing potential of a panel of five different LPS molecules. I have demonstrated that *B. fragilis* LPS is 1500 times less potent at inducing TF than *E. coli* O111:B4 LPS and 3000 times less potent than *E. coli* O18K LPS. From this study it was not clear why the *B. fragilis* LPS was less potent than the *E. coli* LPSs. It is possible that, in contrast to *E. coli* LPS, *Bacteroides* LPS was acting via a CD14 independent mechanism resulting in the activation of an alternative signal transduction pathway for inducing TF antigen. In order to fully test this hypothesis, it would be necessary to carry out these experiments in *ex vivo* whole blood in the presence of a CD14 neutralizing antibody. It has already been shown by Delahooke *et al.*, that monoclonal antibodies to CD14 did not block the TNF-α inducing ability of *Bacteroides fragilis* in isolated human mononuclear leukocytes. It is, therefore, likely that *B. fragilis* LPS will also induce TF via a CD14 independent pathway. This being the case, it is interesting to speculate what this alternative pathway might be. It is possible that *B. fragilis* LPS might induce TF via a different receptor such as the Mac-1 receptor, or via a different intracellular signalling mechanism. Interestingly, in this study I have also shown that in the presence of an excess of *B. fragilis* LPS, the TF antigen inducing ability of *E. coli* O18K LPS is reduced by about 65%. This is in agreement with the observations of Delahooke *et al.*, for TNF-α induction. These observations are particularly interesting considering that *B. fragilis* outnumbers *E. coli* species in the gut by about 1000 fold. If

Bacteroides species are the main inducers of TF in conditions whereby the blood stream is contaminated by faecal matter, greater efforts should be made to target this bacterium with immunotherapy instead of *E. coli*. Furthermore, it is also possible that if the *B. fragilis* LPS has the same inhibitory properties *in vivo* as I have seen in *ex vivo* whole blood, this bacterium could serve an important protective role. The mechanism for this masking of the effects of *E. coli* by *B. fragilis* LPS is unknown. It might be speculated that if *B. fragilis* LPS acts via a CD14 independent pathway, this mechanism, whilst causing the induction of less TF antigen, might also set in motion an inhibitory second messenger system to block the effects of *E. coli* LPS. Alternatively, it is possible that *B. fragilis* LPS might induce the transcription of a repressor protein which blocks the CD14 receptor to *E. coli* LPS. It is also possible that this CD14 independent TF induction might be more susceptible to inhibition by ASODNs. This would also be an important further investigation.

In summary, I have shown that the monocyte is a feasible target cell at which to direct antisense therapy aimed at the inhibition of aberrant TF induction. I have had limited success at inhibition of TF antigen with both published and in-house designed naked ASODNs but these limitations are likely to be surmountable if more time and money were available to carefully design a more specific and bioavailable antisense molecule. Unfortunately, this sort of fine tuning is limited to the pharmaceutical industry. Antisense therapeutics is now a fast moving field with the approval of the first antisense drug 'Vitravene' (for the treatment of patients with cytomegalovirus) taking place in 1998, just seven years after the programme was initiated and nine years after serious investment in the antisense technique began. This suggests that this form of therapy is becoming widely accepted and is entirely feasible *in vivo*. The important point now is to identify novel but sensible target proteins which are easily detected and have a paramount role in disease. I believe that inhibition of the TF protein by antisense therapeutics is well worth further investigation as this protein is suitably poised, on a suitable target cell and has been proven to be instrumental in many hypercoagulative states.

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APPENDICES

APPENDIX A

PUBLICATIONS ARISING FROM THIS THESIS

Cunningham, A.J., McIlroy, J.M., Russell, A., Marquis, J., Grindel, J.M., Ludlam, C.A., Stirling, D. Antisense uptake in myeloid cells is mediated by the C3 complement receptor (Mac-1). Blood 1997; 90(10 pt2 S1):pp4595.

McIlroy, J.M., Stirling, D., Cunningham, A.J., Ludlam, C.A. Therapeutic potential of antisense tissue factor in thrombosis. Thrombosis and Haemosatasis 1997; No.SS ppS842.
This work was presented at the International Society for Thrombosis and Haemostasis biannual meeting 8-12 June 1997.

McIlroy, J.M., Stirling, D., Poxton, I.R., Ludlam, C.A. Comparison of tissue factor induction in monocytes by different lipopolysaccharides. British Journal of Haematology 1999; 105(S1):41.
This work was presented at the British Society for Haematology annual meeting 12-15 April 1999.
Paper in preparation to be submitted to Infection and Immunity.

APPENDIX B

EXTRACELLULAR GEM-91 UPTAKE RAW DATA

		GEM-91 Dose Level (μM)		
Cell Type	**Time(h)**	**0.1**	**1.0**	**10.0**
Total Cells	**0.5**	31.9 ± 10.5	58.5 ± 17.2	425.2 ± 112
	1.0	29.2 ± 5.84	100 ± 30.4	284 ± 65.2
	1.5	17.9 ± 4.75	71.8 ± 12.3	258 ± 63.0
	2.0	11.0 ± 4.10	38.9 ± 11.3	132 ± 41.4
	4.0	5.93 ± 3.33	19.9 ± 1.93	115 ± 29.6
Lymphocytes	**0.5**	0.54 ± 0.55	1.7 ± 0.11	69.3 ± 9.65
	1.0	1.65 ± 0.14	8.43 ± 3.18	76.4 ± 11.2
	1.5	0.01 ± 0.27	2.69 ± 1.27	60.6 ± 8.59
	2.0	0.54 ± 0.41	2.85 ± 0.51	49.7 ± 14.7
	4.0	0.30 ± 0.49	2.11 ± 0.21	56.5 ± 12.4
Monocytes	**0.5**	53.2 ± 9.90	115 ± 31.6	499 ± 70.6
	1.0	55.1 ± 4.34	264 ± 79.5	555 ± 60.8
	1.5	51.4 ± 9.56	229 ± 6.62	531 ± 54.8
	2.0	25.0 ± 12.0	132 ± 20.8	468 ± 54.7
	4.0	16.4 ± 3.89	30.9 ± 20.6	63.5 ± 26.5
Neutrophils	**0.5**	31.3 ± 10.0	58.4 ± 17.0	357 ± 47.8
	1.0	20.8 ± 2.40	99.9 ± 30.5	282 ± 66.6
	1.5	16.9 ± 4.95	71.5 ± 12.2	264 ± 60.8
	2.0	12.4 ± 6.23	38.7 ± 10.9	142 ± 48.1
	4.0	5.93 ± 3.16	20.2 ± 2.26	135 ± 26.3

Append B1 Extracellular association of GEM-91with total leukocytes and lymphocyte, monocyte and neutrophil subtypes at concentrations between 0.1-10.0μM up to 4 hours.
Values shown in the table are expressed in fg GEM-91/cell. Each value represents the mean ± sem in each case, n=3.

INTRACELLULAR GEM-91 UPTAKE RAW DATA

		GEM-91 Dose Level (μM)		
Cell Type	**Time(h)**	**0.1**	**1.0**	**10.0**
Total Cells	**0.5**	17.7 ± 1.35	62.0 ± 11.6	130 ± 12.4
	1.0	31.2 ± 7.66	65.8 ± 8.05	129 ± 34.1
	2.0	62.2 ± 7.61	151 ± 52.3	276 ± 86.6
	4.0	87.5 ± 12.3	280 ± 15.5	458 ± 76.2
Lymphocytes	**0.5**	0.40 ± 0.40	17.3 ± 11.9	20.9 ± 5.20
	1.0	0.60 ± 0.17	15.9 ± 11.4	68.2 ± 20.4
	2.0	0.60 ± 0.30	8.80 ± 1.73	93.8 ± 12.1
	4.0	3.70 ± 3.70	12.5 ± 4.87	89.9 ± 30.7
Monocytes	**0.5**	32.6 ± 4.69	117 ± 22.6	208 ± 3.83
	1.0	40.9 ± 11.3	88.5 ± 7.86	395 ± 73.7
	2.0	62.0 ± 27.0	191 ± 60.6	365 ± 121
	4.0	172 ± 65.5	506 ± 44.1	828 ± 211
Neutrophils	**0.5**	19.2 ± 1.48	64.0 ± 10.0	130 ± 12.0
	1.0	24.4 ± 24.4	80.9 ± 7.28	217 ± 40.7
	2.0	59.1 ± 59.1	196 ± 35.4	363 ± 40.4
	4.0	77.0 ± 9.46	301 ± 1.4	460 ± 56.1

Append. B2 Intracellular uptake of GEM-91 into total leukocytes and lymphocyte, monocyte, and neutrophil subtypes at concentrations between 0.1-10μM up to 4 hours.
Values shown in the table are expressed in fg GEM-91/cell. Each value is the mean ± sem of n=3 in each case.

APPENDIX C

ADDRESSES OF SUPPLIERS

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